

Europe's next big battle must be won

Preparations for Europe's next big battle in 1996 have begun with too much public speaking in France and Germany, and too little concern for the kind of agenda that will make a constructive intergovernmental conference.

WHILE Europe still echoes to the noise of the political battles over the Maastricht Treaty a year ago, the drum-beat warning of the next great confrontation can be heard. Last week, Europe's two heavyweights, Chancellor Helmut Kohl of Germany and M. Edouard Balladur, the prime minister of France, declared themselves on the future of Europe as they see it. Each affirmed the importance of the close understanding between Germany and France that has been the inspiration of the European Community (now the European Union, EU) since the beginning.

Both Kohl and Balladur last week also offered a vision of a Europe fashioned on hierarchical lines, with France and Germany at the centre, or at the top. But otherwise the two visions of Europe are very different. Chancellor Kohl wants the EU to take Central Europe under its wing. M. Balladur is less enthusiastic about that objective, but also uneasy at the pace of integration within the EU on which its federalist members have set their hearts. Both men face elections in the next half year; Kohl in November, while France must hold a presidential election in the spring.

The importance of these developments does not lie simply in the content of what was said last week, but in the decision taken at the end of 1992 that in 1996 there will be another intergovernmental conference (of the kind that gave rise to the Maastricht Treaty) to reach agreement on "ever closer union" in matters of foreign policy and defence. Understandably, Kohl and Balladur will be asked to say what their opinions are on these European issues at the elections ahead of them. They may also have calculated that they can only benefit themselves if they stake their claim on the agenda of the 1996 conference well in advance.

But they are mistaken. The truth about the EU is that if it does not hang together, it will fall apart. Indeed, four years ago, one of the arguments for the 1996 conference was that, without some timetable for closer union, the momentum that Maastricht was supposed to generate would be lost. (The more compelling argument for a conference then is that Europe must work out some way of dealing with matters such as that of the republics of ex-Yugoslavia, which on present form could still be a burning issue in 1996.) What has gone wrong is simply that Maastricht failed to generate noticeable momentum of a unifying character. Most member states were politically enervated by the battle over ratification. Even now, with recession still in the air, there is little sense that the battles were worthwhile.

That is why the agenda for the next conference should be

more carefully prepared than that over which people quarrelled at Maastricht. The impatience of the federalists notwithstanding, the most urgent need is for a modest agenda, but one that is widely discussed in public well in advance. There needs, for example, to be a European policy on immigration, given that residents in one member state can choose to live in another. There needs to be a common policy on Central Europe that overrides competition between EU states for special influence, but which commands general respect. There needs to be a common policy on defence procurement, on which most European governments remain old-fashioned and chauvinistic. And it is high time that the EU dusted off its traditional regime for the regulation of internal trade in nuclear materials, given that next year's review conference of the Non-Proliferation Treaty will (or should) require more vigilance of all its members. That should be enough to keep heads of government occupied for a couple of days, the standard time allocation.

Meanwhile, there is much of a general character that should persuade Kohl and Balladur to rein in speculation about a hierarchical Europe of the kind they advertised last week. The most important consideration is that there may by 1996 be four new members of the EU (Austria, Finland, Norway and Sweden), which can be rushed into new grand designs only at the risk of disillusion. By then, with the recession perhaps finally over, it may also have become apparent that the EU is not the naturally prosperous place its founding fathers imagined it must be, but a place in which unemployment is unacceptably too prevalent and overall prosperity is disappointing. In other words, by 1996, it may well be apparent to Europe's taxpayers that the economic miracle the EU was supposed to engender is still a long way off. Talk of "core" membership will accentuate that impression, with disastrous results. □

Blaming somebody else

Japan needs better arrangements for conducting clinical trials of new therapeutic drugs.

JAPAN'S Ministry of Health and Welfare is treading in the familiar path of bureaucracies faced with calamity within their sphere of responsibility: blame somebody, indeed anybody, else. That is what happened last week, when the ministry penalized Nippon Shoji, the Osaka drug manufac-



turer, for putting on sale last September a drug for the treatment of shingles from whose use 15 people died within weeks of the first supplies appearing in physicians' pharmacies (*Nature* 369, 697; 1994). The penalty is a serious one: Nippon Shoji must suspend all drug production for 105 days, a move that will cost the company many millions of dollars in lost sales. A more apt remedy would have been to suspend the salaries of the ministry's staff for a comparable length of time.

The story is not particularly complicated, but is otherwise strangely Japanese. The drug concerned is the antiviral agent called Sorivudine, offered for sale as a treatment for the adult form of chicken pox caused by herpes zoster and known as shingles. Nobody will dispute the need for treatments for this debilitating affliction, often of the elderly. So far as anybody can tell, it may even be an effective treatment for the affliction against which it was intended. The trouble is that Sorivudine, which is an artificially substituted derivative of uracil, can be fatal when administered together with fluorouracil anticancer drugs.

The formal basis for the penalty now levied against Nippon Shoji is that the company did not adequately warn physicians of the dangers of using the drug in combination with anticancer agents; its warning was not prominent on the packages of the drug, and did not mention fatality as a possible consequence of misuse. The ministry also complains that it was informed by the company of only one of three deaths occurring during the Phase III clinical trials of the drug, all apparently linked with the simultaneous use of fluorouracil anticancer agents, implying that Sorivudine might not have been licensed if the company had been more forthcoming.

That is a lame excuse. Elsewhere in the world, the conduct of the clinical trials of new drugs is organized as a quasi-judicial process in which those responsible are required to keep accurate records of all patients, and to make those records available during the approval process. As a result, it is not necessary to suppose that those applying for approval for a new drug tell the licensing authority the truth or the whole truth; whatever they do, there is a high chance that the records will find them out. Of course, that takes time and skill. The ministry's long-standing fault has been its belief that the job can be done instead at hurried committee meetings bringing together the great, the good and the now-inactive.

It is a great shame that technically skilled Japan can do no better by pharmaceutical technology — and its own people. Yet the remedy has been plain for years. Costly though it will be, Japan needs a drug-approval system comparable in thoroughness with those of the United States and Western Europe. It also needs some way of weaning the Japanese population, the world's big spenders in this field, away from drugs of doubtful therapeutic value (see *Nature* 342, 850; 1989). The ministry's alternative, the scheme for installing a fax network to inform physicians quickly of new information on drugs, is not a remedy but merely a strip of sticking plaster. What Japan needs (and can afford) is a modern means of drug assessment. □

Drugs on the track

Athletics authorities are right to ban performance-related drugs, but should do so more intelligently.

POOR Britain, seemingly always at loggerheads with somebody about something, is now up to its neck in a squabble over the use of hormones for improving athletic performance. The circumstances are these. Ten weeks ago, a random drug test on a British woman competitor at an athletic competition in Portugal revealed abnormally high levels of testosterone (the "male" sex hormone which is also normally present at low concentrations in women). The first analysis has now been confirmed by analysis of a second urine sample by the same laboratory in Portugal.

That turn of events has diplomatic consequences. The British women's athletics team had qualified to compete in what is called the World Cup, arranged for this coming weekend, only because of the performance of the woman now suspected of having taken testosterone artificially; she can run 800 metres faster than most other women. If the finding of improper use were already confirmed, the British team's performance at qualifying competitions would be amended retrospectively, and the team would no longer be eligible to compete. But the woman has exercised her right to appeal, and the British team has decided to compete.

All this is a nonsense, and an avoidable one. There are two cogent reasons why athletes are banned from taking drugs. First, it is a means of cheating. Second, many of the proscribed drugs are dangerous to athletes' health. So how to police the use of drugs in sport? The obvious difficulty is that the temptation to use performance-enhancing drugs must be greatest for the least successful athletes, those struggling to win the attention of amateur athletics clubs to which they belong, and through which they might win fame and (now) fortune. That explains why national athletics associations have taken to random testing.

But they have a responsibility to make a better job of it. For one thing, if there are always two urine samples, why are they not analysed independently at separate laboratories? Second, why is there not a third sample, collected for the suspected athlete's own use? Third, while positive results from tests on well-known athletes are invariably made public, why are not negative results also made public? That way, at least, it would be possible to form an objective view of the prevalence of drug-taking in athletics.

There are also physiological questions to be tackled. Athletics authorities suspicious of dosing for testosterone seek a measurement of the ratio of the active hormone to its precursor, epitestosterone (said by the Sports Medical Centre in Lisbon to have been 42:1, or much greater than the normal). Given the frequency with which these measurements are now made, the athletics authorities are themselves best placed to say what is the expected normal range of values of this ratio in athletes, who may by their training differ from other people. But in the absence of the routine publication of the data, even that is an obscure question. □

Europe stakes a claim to compete in high-performance computing

Paris. For the first time, the most powerful supercomputer in Europe is one that has been designed and built here. Installed last month at the European Laboratory for Particle Physics (CERN) in Geneva, the massive parallel processing (MPP) machine is fuelling Europe's ambitions to become an international player in the high-performance computing (HPC) market.

But if Europe needs reminding that innovative technology alone does not guarantee success, it need not look far. Thinking Machines, the US pioneer of MPP supercomputers, last week filed for protection from creditors under Chapter 11 legislation and fired 140 staff, less than two years after it had seemed poised to oust Cray Research, the company that founded and dominated the HPC industry (see *Nature* 356, 95; 1992).

Size matters too. Although Thinking Machines and Intel's supercomputer division briefly shared the MPP market, they were speedily dethroned once IBM and Cray brought out their own MPP machines. Cray's T3D, for example, has captured 25 orders in just 11 months, according to the company. 'Mean time to vendor bankruptcy' is now a significant criterion for prospective buyers

— and the big fish seem better bets.

This brief and bloody encounter may dampen Europe's enthusiasm for battle, especially as its pretender is a small UK company, Meiko, with sales of only £12 million (US\$18 million) in 1992. But despite its size, the company — which designed CERN's machine along with Parsys (UK) and Telmat (France) — beat off US rivals in a David and Goliath showdown last year to win a \$17 million contract from Lawrence Livermore National Laboratory for its CS-2 MPP machine.

This has lent credibility to Meiko's claim that its technology is internationally competitive. But, according to an official from the European Commission's (EC's) HPC programme, the company is still likely to need massive investment and backup from a large company if it is to succeed. The commission subsidizes research at Meiko, and also bought two CS-2 computers, one for CERN (which is leading one EC HPC project) and the other to be installed at the European Centre for Mathematics in Toulouse in a deal worth around Ecu9 billion (US\$10.97 billion).

Overall, the commission will draw on funds from the 12 member states of the



Meiko's CS-2 MPP supercomputer was recently installed at CERN.

European Union (EU) to spend Ecu240 million on high-performance computing between 1995 and 1998. More than half of this will go, not on hardware development, but on overcoming the lack of software that is the main bottleneck to growth of MPP.

MPP machines share out tasks among hundreds or thousands of nodes, each a relatively cheap chip coupled to a memory. They are faster and cheaper than traditional vector supercomputers, which push data, production-line fashion, along just a few very powerful — but purpose-designed and thus highly expensive — chips.

But coordinating MPP demands new architectures and algorithms, and software written for vector computers, which can be based on decades of work, will often not run on these. Modifying programmes can take years, demand an army of PhDs, and cost more than the supercomputer. Not surprisingly, some users, especially commercial ones, are reluctant to switch to MPP.

This may now be changing. For one thing, the off-the-shelf chips used in MPP have grown faster and more powerful. This means that fewer nodes — and less programming — are needed. Chips have also become more compatible with industry standards, such as Unix.

Some observers, such as Chris Elliot of Smith Systems Engineering (UK), a company that advises on supercomputer purchases, also argue that the main obstacle to parallel programming is cultural, and that this too is beginning to change.

"We've got used to going to enormous trouble making parallel problems into serial ones to run on normal computers, and then complaining we can't run them parallel," Smith says. Investment by large companies in MPP is encouraging users to take the risk to start programming MPP from scratch, he says. "MPP is the future."

"The best opportunity for Europe, and everybody, is to develop and use MPP software," says Chris Willard, manager of ►

Supercomputing a RISCy business?

Paris. Visionary, maverick or dreamer? The jury is out on Stern Systems Computing (SSC) until later this year, when the small French company plans to move its 'breakthrough' supercomputer prototype from the basement of the Advanced Computer Research Institute in Lyon into the cold light of the market.

Nobody can say SSC is a 'me too' company. While the entire supercomputing industry is shifting towards massive parallel processing (MPP), SSC is ploughing its own furrow with a design based on extending Reduced Instruction Set Computing (RISC) — as used in workstations — to high-performance computing systems.

Adrian Wise, SSC's vice-president of sales and marketing, predicts that industry will prefer SSC's machine, a hybrid between MPP and traditional vector supercomputers, because it would be able to run software written for vector machines at the price/performance ratio of an MPP one. He dismisses the industry's claim (see above) that the software problems that have dogged MPP are ending. "I've been hearing that for 20 years, and we'll be hearing it for another 20," he says.

But some analysts are sceptical of SSC's claims. "Anybody can design the world's best supercomputer, getting it to work in the market is another question," says one. "It's another approach, and its very risky," says one official from the European Commission's high-performance computing programme: "it's still at the R&D stage".

SSC will also produce purpose-built systems, whereas the current trend is to use cheaper off-the-shelf chips. Wise admits SSC's hardware will cost more than rival products. But he is convinced savings on software development will more than make up for the difference.

What nobody disputes is SSC's success in raising cash. The European Union (EU) and private investors have poured FFfr315 million (US\$58.9 million) into the company since its startup four years ago, over half from the EU alone. Many ascribe this success to the personality of SSC's founder and chief executive, Jacques Stern, formerly chairman of the French state-owned computer company, Bull. Cray Research and Digital Equipment also hold minor equity stakes in the company.

Declan Butler

►high-performance technologies at the US analysts International Data Corporation (IDC). The hard fact about making MPP supercomputers, he says, is that the market is small, stagnant and overcrowded; and the end of the cold war means that it is suffering from cuts in US defence research.

IDC estimates the size of the MPP supercomputer market to be \$300 million a year, and the entire supercomputer market to be \$2.3 billion, both relatively small figures compared to a total computer market of around \$200 billion. Commercial MPP systems, mostly large servers, are worth another \$300 million annually, and have higher growth potential than research or industrial supercomputers.

Thinking Machines is taking Willard's advice. It is expected to sell its hardware technology to Sun Microsystems, and use the cash to expand development of MPP software. "We were great at doing R&D, but we were just no good at running a business," admits a company spokesperson.

The big question facing Europe, says Elliot, is whether it needs its own supercomputer industry at all. "It is difficult to make an economic case," he admits. Some say Europe would do better to concentrate on using other people's supercomputers to exploit the potential of such machines for improving industrial processes. But others argue that a home industry is needed precisely to provide the expertise to do this — and point to CERN's new machine as evidence of its capacity to do so.

Declan Butler

TI loses patent battle to Fujitsu

Tokyo. The US semiconductor manufacturer Texas Instruments has lost the first round of a court battle against Fujitsu of Japan, which had challenged the US company's claim to a fundamental patent for integrated circuits on the grounds that the patent applies to old technology that is no longer in use.

But Texas Instruments points out that it took 29 years to win recognition from the Japanese Patent Office for its 'Kilby patent', which describes the first primitive integrated circuit invented by Jack Kilby in 1957.

The patent was finally granted in Japan in 1989, long after it had expired elsewhere in the world. The US company has since been earning millions of dollars in licence fees from Japanese electronics companies for the patent, which, because of the delays in its acceptance, will not expire until 2001. But Fujitsu balked at entering into a licensing agreement, and chose to fight Texas Instruments in the courts (see *Nature* 353, 488; 1991).

Last week, the Tokyo district court ruled in Fujitsu's favour. It concluded that two Fujitsu products, a 1-megabit

Engine failure dents outlook for Japan's space industry

Tokyo. Japan's space programme suffered a major setback last week when the first large satellite to be launched by the country's new H-II rocket was unable to propel itself into geostationary orbit because of a faulty apogee engine.

The 2-tonne satellite Kiku (Chrysanthemum) No. 6 cost nearly ¥70 billion (US\$700 million) to build and place in orbit, a figure that includes ¥19 billion for the launch alone.

But Kiku is now drifting in an elliptical orbit, unable to carry out most of the advanced communications experiments planned over a 10-year period. Its failure has dealt a serious blow to Japan's aspirations to enter the market for the commercial manufacture and launch of satellites.

At a press conference held in Tokyo last Thursday, Makiko Tanaka, the director general of the Science and Technology Agency (STA), which is responsible for the space programme, apologized for the failure, particularly given the amount of taxpayers' money involved. But she said progress was impossible without some waste: "I still believe the science is trustworthy," said Tanaka.

Kiku's difficulties stemmed from the failure of a valve in its Japanese-made liquid-fuel engine to open properly, despite several attempts. The valve eventually

jammed partially open, but as a result the engine was only able to achieve about 10 per cent of its required power, and the hefty satellite could not be boosted into geostationary orbit.

The last such mishap occurred in 1980, when a US-made apogee engine on the experimental communications satellite Ayame No. 2 misfired and communication was lost. Ironically, it was partly because of that experience that the National Space Development Agency (NASDA) and Japanese industry joined forces to embark on a strategy for breaking free of dependency on US rocket technology, and to develop the technology needed to launch large satellites domestically, regardless of the cost.

The high costs of such Japanese-made technology — H-II launches, for example, cost nearly twice as much as equivalent launches in the United States, Europe and China — means that NASDA and industry have been stressing the reliability of their technology. But this particular H-II mission has been plagued with a series of technical failures. Indeed, it was only after three attempts that the rocket got Kiku off the ground (see *Nature* 371, 3; 1994).

Kiku's problems have drawn attention to the very high costs of NASDA's space programme. Tanaka, known for her outspoken views, reportedly told the Cabinet on the day after the press conference that "they [NASDA] make huge efforts to get their budget granted, but don't try so hard to use it effectively".

The Science and Technology Agency has requested a large 8.7 per cent increase of ¥183.7 billion in the space budget for the next fiscal year (see *Nature* 371, 3; 1994). Kiku's failure may well adversely affect negotiations with the Ministry of Finance.

NASDA is still planning to attempt some experiments in the satellite's present elliptical orbit. But most of the planned experiments depend on it being placed in geostationary orbit.

One key set of experiments would have involved sending signals from a ground station via Kiku to other satellites in lower Earth orbit. That is now likely to be impossible, given that Kiku will be in constant motion over the Earth.

Despite the failure, NASDA says that future H-II launches will proceed as planned, including the launch of an advanced communications satellite COMETS in fiscal year 1996, which will have a liquid-fuel apogee similar to the one that failed on Kiku. But agency officials do not anticipate any difficulties with this launch, as the structure of the fuel valve to be used is different.

David Swinbanks

Academy boosts US fishermen in tuna battle

Washington. A National Research Council (NRC) report on the Atlantic bluefin tuna seems likely to harden the negotiating position of the United States when it meets with European nations in Madrid in November to discuss the valuable fish.

The conclusions of the study by the NRC — the operating arm of the National Academy of Sciences — provide little comfort for conservationists seeking stricter controls on tuna fishing in the eastern Atlantic, because it found that stocks of the tuna in this area, where quotas are in force, have stabilized over the past six years.

But the report also found that there is considerable 'mixing' between the western stock, which breeds in the Gulf of Mexico, and the larger eastern stock, which is spawned in the Mediterranean. This conclusion (which has also upset conservationists) has been warmly welcomed by US fishermen, who want action to stop the unregulated harvesting of young tuna in the Mediterranean.

The bluefin tuna (*Thunnus thynnus*) is a large and remarkable fish that migrates northwards through the waters of many nations from its spawning grounds. It can grow up to 10 feet in length, and attracts premium prices of up to \$10,000 a fish in Japan, its main market. But immature fish are caught in huge numbers in the Mediterranean, and are subsequently canned as ordinary tuna.

Since 1982, the bluefin tuna has been managed by the Madrid-based International Commission for the Conservation of Atlantic Tunas (ICCAT) as two separate stocks, divided by the 45° longitude, which stretches down from the tip of Greenland. According to US observers, this has led to a tight quota regime aimed at protecting the population in the eastern Atlantic, while fishing of the larger western population continues virtually without restriction.

But the NRC panel, chaired by John Magnuson of the University of Wisconsin, which has reviewed the scientific data, said last week that there is strong evidence of 'mixing' between the two stocks. The panel also found that the decline in the size of the western stock has been halted since 1988.

The panel's report is likely to force ICCAT to abandon plans for tighter quotas for US fishermen in the western Atlantic. "This is the best thing that could have happened," says Richard Ruais, director of the East Coast Tuna Association based in Salem, New Hampshire. "We think the report will be of tremendous benefit to ICCAT and to the fishermen."

Congressman Gerry Studds (Democrat, Massachusetts), who was instrumental in getting government agencies to commis-

sion the report, says he also expects the study to persuade ICCAT to take action to restrict fishing by European fishermen in the Mediterranean.

The US National Oceanic and Atmospheric Administration (NOAA) is now discussing the report's conclusions prior to deciding on what position to take at ICCAT's next meeting in November.

But not everyone in the United States agrees that the onus to conserve the bluefin

servation measures in the western Atlantic by passing the buck to the eastern Atlantic and Mediterranean.

Magnuson characterizes the report as "a correction" to previous assessments. But he says that he does expect it to change the way ICCAT looks at managing the bluefin tuna, and also emphasizes the report's call for research to provide new data on fish stocks.

It is also clear from the report that much of the basic information, needed to determine whether there is in fact one stock of bluefin tuna rather than two, is not available. Scientists do not know whether the two stocks interbreed and what the stock levels are. Furthermore, they have only very hazy estimates of the 'mixing' that goes on across the Atlantic.

"Our analysis says that the population is stable [in the western Atlantic] but we don't know why," says Terry Quinn, a panel member from the University of Alaska. "It is almost appalling that the basic data needed for managing the population is not there."

Quinn's comments raise the issue of how the many governments with interests in a fish such as the bluefin tuna are ever going to agree and enforce management policies on the basis of scientific results that remain so uncertain. "They have to use the best available science," says Magnuson. "Many of the questions which they might put to the scientists cannot be answered. We need better data — but its absence can't be used as an excuse not to use what we have."

Colin Macilwain

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Bluefin tuna: 'one stock not two' in north Atlantic.

tuna should be shifted across the Atlantic. "These are two separate stocks, who breed in separate areas," insists Carl Safina of the National Audubon Society, a powerful conservation group. Jointly with other groups, the Audubon Society has issued a statement branding the report "inconsistent and inconclusive", and claiming that it "should not be used as an excuse to avoid additional con-

Funds flow for eastern gene-banks

Paris. Western aid is flowing into a US\$2.5 million plan, coordinated by the International Plant Genetic Resources Institute (IPGRI) — formerly the International Board for Plant Genetic Resources, based in Rome — to rescue more than half a million varieties of 2,500 plant species at risk in gene-banks in eastern Europe and the former Soviet Union (see *Nature* 360, 201; 1992).

In particular, the United States has donated \$400,000 to help secure the 350,000 accessions held at the Vavilov Research Institute of Plant Breeding in St Petersburg, Russia, and its 17 experimental stations. The United Nations Development Programme has donated \$25,000, while the US Agency for International Development (AID) has pledged \$500,000 and France FF500,000 (US\$93,450).

An immediate need has been to repair long-term storage facilities. In the longer term, says IPGRI, money will also have to be spent on modernizing the institute. Com-

puting facilities will need to be improved, for example, to improve the dissemination of the information which has been carefully compiled and monitored.

Sweden has also donated \$30,000 for emergency repairs to Hungarian gene-banks, and Switzerland \$42,000 for those in Bulgaria. A \$400,000 project, coordinated by IPGRI and the Centre for Genetic Resources in the Netherlands — whose government is paying — will coordinate the development of a common documentation system in seven east European countries.

AID has also pledged \$500,000 to protect botanical specimens at the Komarov Botanical Institute in St Petersburg, whose plight was recently raised by British academics (see *Nature* 371, 9; 1994). For its part, the UK government has donated \$30,000 to duplicate a unique collection of onions and garlic (*Allium* sp.) held in Olomouc in the Czech Republic.

Declan Butler

South Africa 'must fight scientific illiteracy'

Cape Town. The Royal Society of South Africa has suggested that the government should consider some radical solutions to the problem of widespread scientific illiteracy among schoolchildren and adults — including for the first time the introduction in schools of courses in evolutionary theory.

It has also suggested that the country consider increasing its efforts in taxonomy. And that one of its top scientific priorities should be the construction of a new optical telescope — although achieving this will depend on finding international partners.

The suggested priorities are contained in a discussion document on science research policy in South Africa. Apparently timed to influence policy decisions by the new government, the document provides a detailed analysis of the nation's strengths and weaknesses in all fields of science, and the related issue of science education.

The document itself has been drawn up by various members of the society. For example, the president of the society, George Ellis, suggests that schools become involved in advancing science through environmental monitoring efforts, weather observations, accumulating population census data and biodiversity atlas projects.

Two educational projects, aimed at providing comprehensive locality records of birds and the country's spectacular *Protea* flora, have already proved enormously successful in enlisting the help of amateurs.

The document also suggests significant changes in school syllabi, in fields such as mathematics and biology. For example, it specifically recommends placing emphasis on the importance of the theory of evolution in biology, which is currently excluded from syllabi in South Africa. Partly as a consequence of this, biology, despite an abundance of factual material, has suffered from rote-dominated teaching.

Taxonomists are likely to welcome a recommendation in another chapter of the document for the creation of a programme designed to speed up the rate of progress in taxonomy — particularly in botany — to take account of South Africa's unique diversity of species.

This recommendation may be adopted by the new government, which is keen to establish a 'green' image. In doing so, for example, it would honour the country's commitment, made in line with the declaration signed at the Earth Summit in Rio de Janeiro in 1992, to draw up an inventory of its biota.

In terms of international science, one chapter in the report points out that the country's location and climatic conditions makes astronomy an important field to develop. Top priority, it says, would be the acquisition of a new 3.5–4-metre telescope, to replace the existing one at the Astronomical Observatory at Sutherland, about 400 km north-east of Cape Town.

This is unlikely to take place without

foreign assistance. The German government has expressed some interest in erecting a telescope in the Gamsberg mountains in Namibia, and it is also being seen as a possible alternative site for the European Southern Observatory's Very Large Telescope, currently being built in Chile (see *Nature* 368, 676; 1994). But this would involve the duplication of infrastructure that is already in place at Sutherland.

Another controversial aspect of the report is its section on the National Accelerator Centre (NAC), currently run on funds separately earmarked by cabinet (see *Nature* 365, 766; 1993). The Royal Society document recommends that the future of the centre be reviewed by a specialist committee. But it is sceptical of the prospects of obtaining non-governmental funds to operate its cyclotron.

The document describes it as a "cause for concern" that the "powerful phalanx of theoretical nuclear physicists in South Africa has not enthusiastically devoted any significant portion of its attention to research in physics accessible to experimentalists using the NAC cyclotron".

It also questions whether NAC's activities justify a staff of 228, but emphasizes that the centre's R35 million (US\$10 million) budget is negligible compared to that of the Atomic Energy Corporation — whose subsidy has, surprisingly, been increased by the new government, to R509 million.

The document's comments have already had an impact in political circles. Last week, during a debate on science in the new South African Parliament, at least one member referred to the criticisms it makes of the NAC to suggest that the government's investment in the facility be reassessed.

Michael Cherry

Museum seeks £2m for Darwin's home

London. Britain's Natural History Museum is seeking to raise £2 million to restore and develop Down House in Kent (right), home to Charles Darwin for 40 years and the place where he wrote a dozen books, including *On the Origin of Species*.

The museum bought a 99-year lease on the 20-room house early last year from the Royal College of Surgeons, which itself had bought it from the British Association for the Advancement of Science in 1952. Although some of the rooms are currently open to the public, at present only 6,000 visitors make the trip to the site, 15 miles south of London, each year.

Around £500,000 is needed for urgent repairs and restoration of the building, and a similar sum for longer-term improvements. The museum's lease includes the gardens,

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the laboratory in which Darwin carried out many of his experiments and the Sandwalk, an elliptical stand of trees around which Darwin liked to walk when pondering on difficult problems.

The museum is hoping to raise a further £1 million to create a library, exhibitions on the significance of Darwin's work, and educational facilities for school groups, as well as parking and visitors facilities away from the main site.

M. V.

UN agency launches biotech data network

Vienna. The United Nations Industrial Development Organization has introduced an online Biosafety Information Network and Advisory Service, which provides electronic access to a number of databases containing information about biotechnology guidelines, regulations and standards for the environmental release of genetically-modified organisms.

The network provides details on national regulatory authorities worldwide, as well as an electronic gateway to other biosafety-related databanks. A key function will be to help national authorities draw up and implement biosafety guidelines, and the network is also intended to provide industry with information on worldwide regulatory trends.

Patent office braces itself for report on costs and staffing

Munich. The Munich-based European Patent Office (EPO), faced with complaints of excessive costs and centralization, last week extended the term of office of its president Paul Braedli to give itself more time to appoint his successor.

Braedli, who has already served two five-year terms as president, is not standing for a third term as he is approaching retirement age. His appointment, due to expire next May, will instead be extended until the end of 1995, while the EPO council considers three candidates as his successor.

Whatever the outcome, the new president will arrive at a politically sensitive time for the office. He or she will be faced with the results of a investigation currently being carried out by EPO's administrative council, made up of representatives from each of its 17 member states, whose report should be completed by the middle of next year.

The investigation was prompted by the unhappiness of some member states over issues such as the cost of patenting through the EPO, the lower than expected ratio of European to non-European patent applications, and the desire that some of the EPO's activities be decentralized to national patent offices.

Much debate has been generated by the relatively high, tax-free salaries enjoyed by the EPO's 4,000 staff, particularly locally in Bavaria, where politicians have campaigned for an end to the tax-free status of employees. The report may suggest that staff and accommodation costs be reduced over the next few years.

The investigation is also looking at ways of reducing high translation costs that add at least 20 per cent to every patent application. Both issues are politically very sensitive.

It was hoped that applications from Europe would constitute at least two-thirds of the total. In fact these make up only half. One way of improving this ratio may be to decentralize patent information services to national offices.

A. A.

Dutch universities fight plan to cut teaching and research

Munich. The Netherlands could become the first mainland European country to abandon the continental system of university education under government proposals to move to a cheaper Anglo-Saxon system, with shorter undergraduate degree courses.

But Dutch academics are fighting the proposals. They are also resisting the government's efforts to cut back heavily on university and research funding as part of a package of across-the-board cuts intended to trim F118 billion (US\$10.26 billion) from public spending over the government's four-year term of office.

Addressing the opening session of the new Dutch Parliament last week, Wim Kok, the prime minister and head of the country's left-of-centre coalition, announced plans to cut F11.5 billion from the universities budget of the Ministry of Education and Science.

The government wants F11 billion of this to come from the country's generous system of student support.

It argues that this could be done by moving to a two-tier degree system similar to that in the United States and the United Kingdom, in which a three-year bachelor degree is followed by a masters degree only for those students with a specific interest in further studies.

At present, a Dutch degree officially requires four years of study. But students tend to spend up to six years completing a degree course, which includes a significant research component.

The government argues that as well as saving money, the proposed change would give Dutch graduates a better chance of finding jobs outside the Netherlands, as they would leave university earlier and, for example, fit more easily into the job market in the United States.

But the proposal does not have universal support. A meeting of university heads last week expressed alarm at the proposal, warning of the danger of linking academic reform to spending cuts. Such officials argue

that the Anglo-Saxon system is not appropriate to an educational system which guarantees university education to all students with a high-school diploma.

The Netherlands' Research Council (NWO) is also campaigning against the proposal, claiming that it will damage the research potential of the Netherlands by lowering standards of higher education.

The council also argues that the additional cut of F1500 million to universities will mean a loss of 10,000 academic jobs — equivalent to a 15 per cent fall in university staffing — which will inevitably have a direct effect on research.

But the Netherlands is not the only European country to flirt with ideas of academic reform.

Many others, particularly Germany, are starting to believe that the ideal of guaranteeing university education for all high-school certificate holders, with little control over the length of their studies, is a luxury which has no place in the 1990s.

As part of the same austerity package, a further F1700 million is expected to be cut from the research budgets of other ministries during the government's four-year term of office.

Details will be announced by the government on 20 September. But science organizations are fearing the worst. Frans Gubbi, a director of the Dutch applied research organization, the TNO, fears that the government will reduce TNO's basic budget of around F1200 million annually by 10 per cent, a move that could hit environmental and energy programmes particularly hard.

TNO depends heavily on contract research, which brings in around F1500 million annually. Gubbi says that this income will be seriously affected by government cuts to ministries, and that the new cabinet's policy may mean that the organization is unable to keep its basic functions running, with the potential loss of hundreds of research jobs.

The Dutch Social and Economic Council, made up of representatives of industry and the economics community, recently advised the government to raise research spending by F11 billion in order to stop it falling further behind its European neighbours. (Spending on research and development has fallen from a high of 2.3 per cent of gross national product in the 1980s to 1.87 per cent in 1993.)

"Instead they are cutting back further," says Gubbi, who is organizing a major lobby of ministers, civil servants and parliament during the budget debates over the next two months in an attempt to reverse government policy.

Alison Abbott

Research to benefit from ex-communist funds

Munich. Science in the former German Democratic Republic is set to benefit from a share of a large fund which has finally been released from the coffers of the PDS (Partei des demokratischen Sozialismus), the official successor to former East Germany's communist party.

The German government had been trying to get its hands on the money since reunification, arguing that the money had been wrongfully accumulated. Last week, after several months of negotiation, the

PDS agreed to release DM1.1 billion (US\$705 million) to support the 'cultural base' of the new *Länder*.

DM150 million of this sum has been allocated to science, half of which will be distributed by the five new *Länder* governments. The rest of the money is being used by the federal government to support the development of new products in the *Länder*. A call for research grant applications was put out last month in anticipation of the settlement. □

Indian universities 'in crisis' over government funding cuts

New Delhi. The vice-chancellor of Delhi University, Upendra Baxi, resigned last week in protest over government cutbacks which, he claims, have left him with insufficient funds for university maintenance, let alone for research.

Baxi's move has turned a spotlight on the acute financial crisis facing India's 144 universities. Many leading educationalists are worried that the quality of India's science and engineering education will suffer because of the financial pressures that the universities are now experiencing.

Of the 10 centrally funded universities that receive their budgets from the University Grants Commission (UGC), Delhi University gets the maximum possible government support. Baxi's departure because of the "economic bankruptcy" of his university has not only highlighted its own problems but also raised questions about the plight of universities who receive only meagre grants from the UGC.

Delhi University received Rs300 million (US\$10 million) from UGC this year, compared with the Rs450 million that Baxi claims is needed just to pay staff salaries and maintain existing services. Earlier this year a government-appointed committee decided that the university, which has 189,000 students, needed a minimum of Rs24,000 per graduate student. But the UGC funding provides only Rs6,000 per student.

The impact of the cutbacks is being widely felt. According to Baxi, for example, the science library subscribed to 795 foreign journals in 1992, but this year has only been able to pay for 275 subscriptions. Similarly, students found that there were no chemicals available when they came to take practical examinations.

About 4.65 million students attend 7,120 higher education colleges in India, accounting for 10 per cent of the government's total education budget (which is 3.5 per cent of the national budget). Increasing demands on the higher education budget from primary and secondary sectors have forced the UGC to freeze maintenance grants at 1991 levels since 1992, and vice-chancellors have been asked to find alternative sources of funding.

Tigers under threat

London. The continued use of tiger bones, skin and other body parts in traditional Chinese medical practices has produced a "catastrophic" loss of the animal and an urgent need of measures to save the species from extinction, according to a report *Killed for a Cure* due to be published next week by the World Wide Fund for Nature. □

To offset the government cutbacks, for example, the Indian Institutes of Technology (IIT) have raised tuition fees, particularly for foreign students. They have also carried out contract work for industry, and faculty members are allowed to offer consultancies on condition that they give a percentage of their earnings to the institutes.

But universities have been less successful than the IIT in generating income from non-governmental sources, in spite of the fact that, since 1992, private donations have been exempt from income tax. Raising tuition fees in the case of universities is a politically sensitive issue, and Baxi said

He was on his way to serve the drinks at a party on our lawns, when the official rickshaw had a puncture, and something must have finally snapped...



even such a move would not be sufficient to resolve the current crisis.

For example, the Bangalore University has reacted to its financial difficulties by diverting money from its student welfare fund, and reducing the intake of students on 'capital-intensive' courses such as microbiology and computer science.

One vice-chancellor in Bihar state commutes to work in a cycle rickshaw because his university cannot afford petrol for the official car. Magadh University, also in Bihar state, last year paid Rs1.5 million on interest payments on its bank overdrafts.

Some colleges in Delhi have been earning money by hiring out their lawns for marriage parties, while Calcutta University has decided to sell more than 950 gold and silver medals that have been lying unclaimed since 1906.

Baxi claims that if the financial situation facing Delhi University does not improve in the near future, "its soul will be lost for ever". Administrators of other universities, trying to survive in the face of the same pressures, hope that Baxi's sacrifice will not have been in vain. **K. S. Jayaraman**

Science loses its male image among UK schoolchildren

London. Survey results released in London this week show that most schoolchildren acknowledge that science and technology are important in their everyday lives — in particular, science's contribution to new technologies such as computers and television — but think that it should be more fun.

At the same time, while the majority of parents and teachers still perceive science as a male domain, with 80 per cent of female teachers believing this, pupils themselves (and particularly girls) are less likely to agree.

The survey was timed to coincide with the launch of the Institution of Electrical Engineers' 70th Faraday Lecture, and was commissioned by the telecommunications companies Motorola and Cellnet as part of a £1-million sponsorship of the lecture.

It showed that, as students embark on GCSE courses taken usually at the age of 16, their attitudes change; 32 per cent of 13-year-olds thought science was fun, and 72 per cent that it was useful, but these figures fell to 23 and 63 per cent respectively for those one year older. Conversely, the number of children who considered science boring and difficult rose between these two ages.

Commenting on the results, Jill Nelson, head of science promotion at the Royal Society, said they may reflect the process of pupils "getting down to serious study" associated with examination courses, and might also apply to other subjects.

But she welcomed indications that girls had not been turned off science and technology, and did not see science as a subject closed to them. This is despite evidence from Royal Society studies of "the damage being done much earlier", with many girls being put off science as early as primary school level.

The Faraday Lecture is held annually with the aim of encouraging schoolchildren to pursue careers in science and technology. The travelling lecture begins in Glasgow in September and finishes in Brighton next March, after visiting 18 cities in the United Kingdom and Ireland.

The findings are the result of a survey of more than 1,500 13-16-year-olds across the United Kingdom, 185 parents and 100 teachers of biology, chemistry and physics. The children answered a self-completed questionnaire, whereas parents and teachers were interviewed.

Teachers tended to assume that students would find science dull, and stressed the benefits of studying it for future career prospects rather than because it was enjoyable or fun. And 32 per cent of students said that science should be made more fun, with more emphasis on an interactive approach and less emphasis on textbooks. **Maggie Verrall**

Exxon Valdez and bioremediation

SIR — In response to the report by Bragg *et al.*¹ on bioremediation of the *Exxon Valdez* oil spill in Prince William Sound, Alaska, in 1989, Henry Miller² deplores the regulatory climate which he feels is responsible for suppressing the use of genetically engineered microorganisms (GEMs) for cleaning-up environmental contamination. However, stringent regulations are not the issue here. The real problem lies with GEM technology itself.

The concept of using genetically engineered 'super-bugs' to degrade something as complex as crude oil is seriously flawed. Crude oils are mixtures containing several hundred components including normal and branched-chain alkanes, substituted and unsubstituted cycloalkanes and mono- and polyaromatic hydrocarbons along with the so-called NSO (nitrogen, sulphur and oxygen containing) compounds such as cresols, pyridines, quinolines and benzothiophenes. The metabolic potential required to deal with this diverse array of chemical structures is considerable. Even if it were technically feasible to incorporate all the necessary genetic information into recombinant microorganisms, the burden of maintaining all these genes is likely to be so great as to make the recombinant strains noncompetitive in the natural environment. It is simplistic to think that introducing just a few genes (the limit of today's technology) into an organism will create a 'super-bug' that can degrade crude oils single-handedly. This approach was tried in the mid-1970s (ref. 3), but no commercial products were forthcoming, primarily because crude oils are very effectively biodegraded by communities of naturally occurring indigenous microorganisms⁴.

Miller's justification for wanting to use genetically engineered microorganisms is that "naturally occurring microorganisms seem not to be up to the job" (of bioremediation). This is patently not true for crude oil and many organic contaminants in the environment. We have yet to come across an environment (soil, ground water, surface water, shoreline and so on) contaminated with petroleum hydrocarbons where a competent population of hydrocarbon-degrading microorganisms capable of cleaning the environment did not exist. To the best of our knowledge, this is also the experience of others working in this field⁵. On many occasions, cleaning takes place without the interference of man (natural attenuation)⁵. However, the biodegradation process is often limited by the size of the active microbial population that can be dependent on available mineral nutrients, such as nitrogen and phosphorus. Biodegradation can then be speeded up by the use of fertilizers, as Bragg *et al.* have demon-

strated. Genetically engineered microorganisms are no different from wild types in their requirements for, among other things, nitrogen and phosphorus for growth.

Miller describes the Exxon approach to bioremediation as the "technology of the nineteenth century". Admittedly, "slopping" fertilizer on a beach to promote microbial activity does not have the 'high-tech' sparkle of GEM technology. However, these natural tools do work and do lead to cost-effective solutions to many of our environmental contamination problems. We should exploit them.

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Bad example

SIR — Dr Kary Mullis, who won the Nobel prize for chemistry in 1993, was invited to speak at the 28th Annual Scientific Meeting of the European Society for Clinical Investigation in Toledo during April. Just before the lecture, he told me he would not speak about the PCR but would tell his ideas about AIDS not being caused by the HIV virus. His talk was in style rambling and in content inappropriate for a public appearance of a leader of science, especially with several hundred young scientists present. His only slides (on what he called "his art") were photographs he had taken of naked women with coloured lights projected upon their bodies. He accused science of being universally corrupt with widespread falsification of data to obtain grants. Finally he impugned the personal honesty of several named scientists working in the HIV field.

His own explanation of the immunodeficiency syndrome was incoherent and insubstantial. As chairman, I stopped the lecture after half an hour and asked him to apply the scientific method to the problem, asking him to answer three specific questions about the transmission of AIDS to haemophiliacs and from mother to child. His reply was again inappropriate both intellectually and in style.

Mullis several times insisted that having

won the Nobel prize gave him authority to speak. Surely the credible authority of a Nobel laureate should be confined to the subject for which he won the prize (in Mullis's case, chemistry). Mullis not only decreased the nobility of the prize but his attitude was, I believe, a potential corrupting influence on young scientists: among other things, for example, he claimed himself to have changed data-points so as to make data-sets appear more significant by way of illustrating that the practice is a common one.

The council of the European Society for Clinical Investigation will not be inviting Mullis to speak at further meetings.

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Strange but true

SIR — In a recent issue of *Nature* (370, 107–8; 1994), Arthur C. Clarke described as "truly incredible — one might almost say eerie" the fact that the impacts of the largest fragments of comet Shoemaker-Levy 9 coincided with the twenty-fifth anniversary of the Apollo 11 Moon landing. The fact that the impact of the largest fragment coincided, almost to the minute, with the twenty-fifth anniversary of the landing is actually only the centrepiece of a wider and more incredible set of coincidences: the first fragment of the comet hit Jupiter on 16 July (twenty-fifth anniversary of the launch of Apollo 11), and the final fragment hit on 22 July (twenty-fifth anniversary of Apollo 11's departure from Moon orbit). So the start, climax and end of the series of impacts coincided exactly with the start, climax and end (in the sense of departure from the Moon) of the Apollo 11 mission to the Moon.

A recent television programme informed me that the first SL9A impact, on 16 July, hit Jupiter at 4.18 p.m. US time (I am not sure which time standard that was). Today, I looked up the timings of the Apollo 11 mission in an encyclopaedia, and found the time of the Moon landing quoted as 4.18 p.m. US Eastern Daylight Time. I am a lifelong sceptic, but these coincidences interest and indeed startle me. I would like to invite those with easy access to the precise timings of all 21 cometary impacts, and the precise timings of key events of the Apollo 11 mission, to examine carefully just how "incredible" and just how "eerie" the coincidence is.

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Advantages of general practice

SIR — Barbara J. Culliton argues that a 50 per cent quota of medical students earmarked for general practice is unlikely to help the US health-care crisis (*Nature* 370, 501; 1994). Her damaging allegations about health systems based on primary care must not go uncontested.

I challenge her to cite valid epidemiological references to support the claim that because of a lower proportion of specialists, "people in Britain are more likely to die of cancer than their counterparts in the United States and Europe". Any such 'evidence' is more likely the result of inappropriate 'screening' leading to earlier detection but no benefit (and in some cases discovery of disease that would never be clinically manifest). Enhanced 'survival' entails living longer with the diagnosis rather than more effective treatment or real extension of life. Her figures presumably also omit the millions in the United States excluded from the 'skilled specialist' Utopia because of inadequate insurance cover (U. E. Reinhardt, *New Engl. J. Med.* 330, 1452–1453; 1994).

More importantly, she neglects the role of the primary-care physician in selecting the specialist a patient sees. A subject with abdominal pain might refer herself to an accident and emergency department, urologist, gynaecologist, venereologist or gastroenterologist, and receive different high-technology investigations from each. Even if the specialist rapidly recognizes a condition outside his or her field, appropriate referral is delayed. Similarly, if an uncomplicated urinary tract infection were the cause, the primary-care physician could make the diagnosis sooner, and be capable of prescribing appropriate therapy at a fraction of the expense.

More insidiously, the 'worried well' or those with benign self-limiting conditions are subjected to stereotypical 'full diagnostic work-ups'. The nature of many of these investigations, validated only in the severely unwell, yields a steady supply of borderline abnormalities, incidental findings, false positives and artefacts. These in turn ensure further rounds of interventions.

The patient is protected from this if seen first by a primary-care physician with a broad knowledge of the patient's medical history and economical, emotional, psychological or spiritual backgrounds of which Culliton speaks so disparagingly. She shares an attitude born of uncritical attendance at too many medical school case presentations; there symptoms are inevitably due to esoteric yet highly treatable organic conditions missed by blundering generalists. I suggest it is within those ivory towers that she should look for the "physicians as God" she cites, convinced of the omnipotence of high-technology medicine. Primary care physi-

cians are best placed to ensure simple preventive measures and behavioural changes. These can save and enhance far more lives than continued overprovision of specialists, many of whom make little or no contribution to basic or clinical science.

Research suffers on both sides of the Atlantic from administrations bent on short-term minimization of cost rather than long-term maximization of value. But Culliton has done the biomedical community a disservice with her transparently biased and elitist rhetoric.

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SIR — Culliton, in arguing that primary care is not the answer to the health-care problem in the United States, fails to mention an important fact. Total spending on health care in the United States is about 14 per cent of gross domestic product (GDP) and about \$2,000 per capita, yet at least 35 million people have no cover. The author contrasts the system in the United Kingdom unfavourably with that in the United States. In the United Kingdom we spend 7 per cent of our GDP (about £600 per capita) and there is complete coverage of the population.

Health care in the United Kingdom seems to be more 'efficient' than that in the United States even though it is hampered by inadequate funding. A major difference between the two systems is that in the United States the patient must perform a preliminary diagnosis and seek out the appropriate specialist. Consideration of Bayes' theorem indicates that the predictive value of a test depends upon the prevalence of the condition in the population (T. Bayes, 'Essay towards solving a problem in the doctrine of chances', *Phil. Trans. R. Soc.*, 1763.) A specialist who is used to seeing a population in which the proportion of people with coronary artery disease is high will thus tend to overdiagnose the condition when exercising the same clinical skills upon a population where the prevalence of disease is low. This is because specialists' clinical judgement and the tests upon which they rely are 'tuned' to the population they normally see.

A man who is depressed and worried about a muscular pain in his chest may choose to see a cardiologist who diagnoses ischaemic heart disease. The patient may then have further tests that lead to major heart surgery. The operation may be expertly performed and technically very successful, but, if the main problem of depression persists, it is difficult to argue

that the man has received "high quality health care".

Human beings are more than the sum of the functions of their limbs and organs. Many consultations with physicians have a psychological or social component that presents as physical illness and can lead to unnecessary or harmful investigation.

Comparisons between the health-care systems of different countries are fraught with problems. Health has less to do with doctors and health care than many imagine, and more to do with the social and economic circumstances of the individual. But by most parameters the UK health-care system ranks highly and the relationship in the National Health Service between primary and secondary care probably deserves some credit for this.

Modern biomedical research is essential and we should be quick to evaluate it and make its benefits available to the general population. The problem with the UK system of health care is not with its structure but with the lack of funding. It is most efficient to use specialist resources on a population where the prevalence of 'real' illness is high. This means that the doctor the patient first sees must be expert in identifying those who will benefit from specialist help. A well-educated, well-supported primary-care physician is the key to the efficient delivery of high quality, high technology health care.

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Apoptosis: two p or not two p?

SIR — Attention is being paid to the correct pronunciation of the coined word apoptosis, for programmed cell death. Clearly the 'p' in ptosis is silent, and on this basis students are commonly exhorted to pronounce apoptosis as apo'tosis.

The silent 'p', however, appears neither correct nor attractive in words in which the Greek-derived 'pt' occurs in the middle of a composite word. Consider, for example, two words containing the Greek word for wing (pterodactyl, helicopter), in which the 'pt' is pronounced quite differently. In Italian, by contrast, 'p' is neither written (nor sounded) in the equivalent words.

This suggests that students who sound both 'p's in apoptosis may be correct, and that adenoidal attempts by their elders to suppress the second 'p' are not well grounded in etymology.

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Improving Indian universities

SIR — The problems of Indian universities (*Nature* 366, 617; 1993) are much more complex than those experienced in Russia and elsewhere. What many Indian universities urgently need is better leadership and less political interference.

In many parts of India, teachers in junior colleges are better qualified than senior college (graduate and postgraduate level), teachers. To teach in a junior college, one needs a relevant postgraduate degree (MA, MSc, MCom) and a degree in teaching (BEd), whereas in senior college one needs only a postgraduate degree. If a teaching degree were an essential qualification, teachers would be much more effective. The national entrance test (NET) for eligibility for lecturership (assistant professorship) in the universities and colleges is not compulsory in some places, so there is no uniform standard in higher education. To improve college-level teaching, the government must make the NET and teachers' training compulsory at the senior college level. Around 1980, a research degree (MPhil/PhD) was made a prerequisite for annual increments in pay after eight years of service. For this a faculty improvement programme (FIP) was introduced by the University Grants Commission (UGC), and serving college teachers were given study leave to pursue a research degree. But this has not produced the desired results, perhaps because research experience in a narrow subject area is not what is needed for teaching at college level. The money spent on FIP could have been better used in improving facilities so that students completing research degrees could continue research at their own colleges. The UGC could draw up a fresh programme for library and laboratory improvements in the colleges, which would benefit both teachers and students. A shorter teacher-training programme could be introduced, incurring less expense and bringing a qualitative change.

Because of the shortage of funds, it is essential that available funds are used properly. A committed leadership would help enormously. There are nearly 50,000 unemployed PhDs in India, and what is needed is job oriented courses, vocational guidance and increased fees for college and university education. To avoid excluding children of poor parents from higher education, it would be sensible to increase fees to a quarter of the actual cost and to provide the present level of subsidy only to the genuinely deserving.

In India, teachers ('gurus') are traditionally respected next only to one's parents. That is one reason why many choose teaching as a career. The salary of a junior staff member in a university department is roughly US\$150 a month

(\$75 as basic pay and a similar amount as perks). A full professor may get roughly double that. (The figures are twice those reported in your article.) Difficulty in finding suitable accommodation is not the only reason why academics tend to stay where they are. India is a country of many languages and many cultures. Each state has its own language and culture. Those seeking jobs and those looking for suitable candidates probably have a bias towards people of similar background. The situation could be improved by making appointments at national level on the basis of a competitive examination (as is done with central civil service and bank jobs).

Universities need more autonomy, but it needs first to be established whether or not the autonomy enjoyed at present is properly used for academic growth. As long as universities are dependent on state governments for funds and there are no reliable and scientific methods for evaluating their performance, more autonomy may lead to more problems.

A distinction needs to be made between the academic and administrative functions of the universities. The examination departments should be brought under an administrative head and also under the consumer protection act to make them more efficient. A uniform standard at national level could be brought about by introducing a common syllabus and a common body for the coordination of examinations in all universities.

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Animal welfare

SIR — The five letters in the correspondence section (*Nature* 369, 9–10; 1994) concerned with Peter Singer's review of the books *Monkey Business* and *In the Name of Science* (*Nature* 367, 523; 1994) all serve as examples of the 'we/them' attitude that sadly characterizes the approach taken by many people interested in animal welfare.

Adrian R. Morrison wrote in a dispassionate manner about the limb injuries to two monkeys and claimed that they were "in otherwise good health", using criteria ("alert, well-fleshed and with gleaming hair coats") that in no obvious way indicate either good health or the absence of pain and suffering. Morrison also felt comfortable commenting on Singer's "inaccuracies and apparent lack of medical knowledge", supporting his claim by citing a paper by Sharon Russell and Charles Nicoll (*Proc. Soc. exp. Biol. Med.*; in the press), the purposes of which

are to cast doubt on Singer's character, his position on animal welfare taken in his book *Animal Liberation*, and his citations or interpretations of a very small percentage of articles to which he refers in chapter two ("Tools For Research") of *Animal Liberation*. However, Morrison failed to point out (and perhaps did not know) that Russell and Nicoll revised their prepublication draft after discovering that they were in error about one of their major beliefs concerning Singer's intentions to mislead readers of his book, namely Singer's claims about statements made by Elizabeth Whelan, executive director of the American Council on Science and Health. Many of Russell and Nicoll's other concerns about Singer's character, his errors in citation and interpretation and his lack of medical knowledge are also questionable as is shown in Singer's response to their charges (*Proc. Soc. exp. Biol. Med.*; in the press). People are urged to read both papers carefully to develop an informed view of the nature of the continuing controversy between Russell and Nicoll and Singer, for there are two sides to all stories.

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Population growth

SIR — Orrego, Marengo and Bull (*Nature* 370, 92; 1994) demolish most effectively the crude application to the present of the Malthusian hypothesis. They show convincingly that there is no significant negative correlation between population density and wealth per head.

Interesting as this is, it has little if any relevance to present concern about population growth. Indeed, the link between poverty and population growth is quite striking. In no OECD ('wealthy') country does the rate of increase per year of natural population exceed 1.1 per cent, and in most of them it is below 0.8 per cent. In most developing ('poor') countries it exceeds 2 per cent and in many is 3 per cent or more. The rapidly industrializing countries of East and South-East Asia, as also Argentina, Brazil and Chile, lie in the 1–2 per cent band.

Although such a correlation does not establish a causal link, it is worth noting the momentum of the growth rate of population. With rapidly increasing numbers, a high proportion of people are in the fertile age groups or younger. Even if family sizes were to shrink immediately to those common in OECD countries, therefore, high growth rates of the populations would persist for many years.

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Making medicine more scientific

Howard Hiatt and Lee Goldman

Modern biology has far-reaching implications for medicine. But a new type of medical training will be necessary if advances in scientific understanding are to become advances in treatment.

MEDICAL school research and curricula in the United States and elsewhere have been profoundly changed by the revolution in biology. Our understanding of the causes of heart disease, cancer and a range of other illnesses is rapidly deepening, and our capacity to prevent and treat some is improving. Many clinical departments have physicians who are well trained in biology as well as patient care, and who conduct sophisticated basic research while continuing to teach younger colleagues and medical students. Little wonder that the era of scientific medicine is said to have arrived.

Unscientific

But has it? Many of these same faculty members, through no fault of their own, continue to practise medicine in ways that can hardly be termed scientific. All physicians must cope with inadequate data about the risks and effectiveness of many of the procedures they perform. Many have received little formal training to help evaluate the information that does exist, and are not as well prepared as they might be to help their patients make decisions.

With a few exceptions, the research of academic clinical departments until recently focused almost exclusively on the biology of disease. The resulting delays in applying the so-called 'evaluative clinical sciences' — including statistics, epidemiology, decision analysis, cost-effectiveness analysis, health-services research, economics, ethics and computer sciences — have led to major problems in advanced health-care systems. These subjects have so far had little impact on the decisions of individual physicians, and even less on national health policy.

Yet if this movement is strengthened, quality of medical care will be greatly improved. Accurate assessment of the cost-effectiveness of interventions may have important effects on health-care reform. And in time, academic departments will acquire a body of individuals equipped to realize the benefits of these skills in the clinic and in teaching.

Since the Second World War, an appreciation of the far-reaching medical implications of the new biology, combined with generous financial support from the National Institutes of Health, has led to a new climate in the clinical departments of many leading US medical schools. But faculty members and students were not trained to apply such advances to

the health of the population. Even population-based studies of medical practice and disease were felt to lie on the periphery of academic medicine. And studies of disease natural history, diagnostic and therapeutic outcomes and systems approaches to the quality of medical care had little or no place on the research agenda. Even such crucial areas as primary care were considered to be intellectually unchallenging and received scant attention. Other important issues, such as the effects of treatments on the quality as well as the length of life, the role of patients and their families in decisions about treatment, the importance of non-physicians in care and the cost of interventions received even less attention.

The medical neglect of the evaluative clinical sciences also worked in the other direction; many key statistical, economic, ethical and other concepts are only now developing in response to medical problems. So for decades biostatisticians and epidemiologists have concentrated largely on public health rather than individual patients.

In the past, the adoption of changes in diagnosis and therapy without rigorous evaluation helped to overwhelm the health-care system with options. Experts to help patients and policymakers select from among worthy choices on a rational basis are essential.

New methods have already markedly improved our capacity to evaluate patients, diseases and their management. The randomized clinical trial, virtually unknown a generation ago, has been refined to the point where tens of thousands of patients in many nations can be studied simultaneously. New statistical approaches often allow results approximating those from trials to be extracted from routine clinical data. And improved measures of health status, cost and quality of life have facilitated research into conditions affecting morbidity.

In the United States, some of these developments have been supported by the federal Agency for Health Care Policy and Research. Yet overall, the funds allocated to such research are but a small fraction of those for more traditional biomedical science.

More than 20 years ago, the Commonwealth Fund, the Robert Wood Johnson Foundation and the Henry J. Kaiser Family Foundation introduced successful

training programmes in the social and evaluative clinical sciences for physicians. At Brigham and Women's Hospital, Harvard Medical School and the Harvard School of Public Health, our two-year Program for Training in Research in Clinical Effectiveness has built upon this experience to become one of the largest of its kind. Begun in 1986, it has grown rapidly (and unexpectedly) from 8 participants in 1986 to 89 in 1994.

The programme begins with intensive courses in biostatistics and epidemiology, as well as electives in decision sciences, health services research, health policy, quality improvement and public health. The courses are designed for physicians concerned with patient care rather than traditional public health issues.

All trainees do one or more research projects. Topics have included the cost-effectiveness of thrombolysis after acute myocardial infarction, risk factors for the development of kidney stones, a randomized trial of aspirin in angina, the role of history and physical examinations in diagnosing carpal tunnel syndrome, the use of blood cultures in diagnosing bacteraemia and the role of surrogates in decision-making for mentally incompetent patients.

Initial reluctance

Of the first 69 graduates, 58 (84 per cent) are in academic medicine. Many fellows also take advanced courses at the Harvard School of Public Health. To begin with, many department chairs appeared surprised, even dismayed, that able young colleagues had chosen to pursue 'clinical effectiveness' rather than 'real' (biological) research. Some physicians were compelled to ask that the contents and goals of the programme be explained to their chairs before sponsorship for them was forthcoming.

The enthusiasm and success of the early graduates, however, appear to have increased interest both within their own departments and elsewhere. Several chairs who had originally agreed to sponsor one person with reluctance have themselves now established units in clinical effectiveness. □

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Origin of the first cell membrane?

Imaginative organic chemistry points the way both to the evolutionary sophistication of the mechanical elements of the cell membrane and to a model for the first synthesis of the archeobacterium membrane.

THE question of the origin of life can be broken into several parts. The most elementary requirement is for self-replicating molecules of some kind, but the most successful of them can ensure only that the whole of the available raw material is turned into material of the same kind when, at least in a closed system, synthesis will stop. That, in itself, is not life, but homogenized death.

That is not to diminish the importance of the search for self-replicating molecules of, say, RNA by people such as Orgel (at the Salk Institute), but merely to remark that living things as we know them are self-replicating entities, each embodying a distinctive blend of chemical components and separated from their surroundings (including other living things) by an external membrane. There have recently been some intriguing experiments showing that such systems can be fashioned from quite simple materials (see *Nature* **363**, 205; 1993).

The obvious difficulty is that there is no obvious connection between a demonstration that membranous vesicles can replicate under laboratory conditions and the course of events on the surface of the Earth more than 3 billion years ago. But those events may be more accessible than we suppose. So much is evident from the biomolecules whose structure is strongly conserved across species; if the nucleotide structure of ribosomal RNA molecules seems everywhere to be the same, it is a fair guess that it has been like that for a substantial part of 3 billion years.

Comparisons between the chemical constituents of different species may be even more revealing. Indeed, there is much in the view that a good deal could be learned about the origin of life by a sufficiently diligent hunt through the literature (*Nature* **367**, 409; 1994). That may not be the stuff of which mega-dollar grant applications are made, but there is now a neat illustration of the value of an appropriately archival approach to the subject.

Writing in the first issue of the new journal *Chemistry & Biology* (1, 11–23; 1994), Guy Ourisson and Yoichi Nakatani, from the CNRS laboratory for the organic chemistry of natural products at Strasbourg, reckon that they have been able to infer, from the constituents of the membranes of extant cells, that terpenoids were plentiful among the constituents of the first cell membranes. It is only proper to remark that this is not armchair science; the authors have been looking for pointers to experiments that may be carried out.

This is how the argument goes. We are
NATURE · VOL 371 · 8 SEPTEMBER 1994

now told at school (right?) that cell membranes are really double layers of phospholipid molecules, with polar phosphate groups on the external and internal surfaces and the lipid (hydrocarbon) parts of the molecules lying in the interior of the membrane. Experiments show that, in the right conditions of pH, phospholipid molecules will indeed assemble spontaneously into pieces of membrane.

But that is only part of the story. For mechanical reasons if no others, membranes must be reinforced by other polar molecules incorporated in their structure. In the membranes of eukaryotes, cholesterol is the most common reinforcing molecule. Ourisson and Nakatani start with the remark that this general rule applies neither to bacteria nor to archeobacteria. Instead, cholesterol is replaced in bacteria by hopanoids (substituted five-ring structures with four cyclohexanes and a furane welded together). And archeobacteria do not have simple phospholipids at all, but molecules on which phosphate groups are anchored to the two ends of a branched hydrocarbon structure through ether bonds and glycerol residues, so that (with the hydrophilic ends being pulled in two directions) they need no reinforcement.

Part of the interest of this tale is that traces of these molecules are common among the compounds of high molecular weight recovered from sediments and from petroleum. The hopanoids, for example, are closely related to the molecule called isoarborinol, another five-ring structure, which was first isolated (by Ourisson and a colleague) in 1969, and only afterwards (in 1989) recognized as a constituent of higher plants, but now inferred to be a constituent of an aerobic microorganism. Organic chemists with time on their hands (if there are such people) might do worse than mount a copy of Ourisson and Nakatani's table of "The terpene fossil record" on their office wall in the hope that it will suggest other connections.

Not that Ourisson and Nakatani are short of ideas. Among other things, they construct a tentative evolutionary hierarchy for the terpenoids in their fossil record. The starting point, they say, must have been some means of making isopentenol units, which are simple 5-carbon mono-alcohols which are then polymerized (perhaps by anchoring one end to a solid surface through a phosphate group). That is certainly one way of making long molecules with double bonds between adjacent carbon atoms in one position out of every four along the chain, with branching

connections at similar intervals.

Figuratively, at least, that provides the outline of a mechanism by which the first membranes might have arisen. There is a solid surface capable of binding isopentenol condensed with a phosphate group through the polar head, and some means by which further isopentenol units can be condensed at the growing ends of the molecules. Given a degree of uniformity in the speed of growth, the time will come when the molecules are just long enough to form a piece of membrane, whereupon the structure will simply peel off the surface in a kind of phase transition, enclosing whatever materials lie within reach in a primitive vesicle of a kind.

The captured contents would almost inevitably include the chemical components required to synthesize the isopentenol units themselves. In real life, so to speak, it would presumably have been a matter of chance whether a vesicle destined to be a kind of archeobacterium would contain genetic elements capable of directing its further replication.

This, it should be remembered, is not just armchair speculation. Ourisson and Nakatani make no secret of their goal, the design of further experiments. It will be interesting to see how soon they can design and demonstrate a catalytic solid surface from which pieces of primaevial bacterial membrane peel off at regular intervals.

But the manufacture of the ingredients of the membranes of archeobacteria is not the only goal. The polymerized isopentenol units provide just the kinds of structures needed to form (by cyclization) the polycyclic terpenes. So there is a prospect of being able to use the mechanical reinforcing molecules in the membranes of bacteria and eukaryotes as a means of telling when, in the course of evolution, the synthetic pathways required for making more complicated reinforcements were recruited to the biochemical repertoire. The outcome of that hierarchical exercise will be interesting and important in its own right.

The lesson to be learned from this is one familiar to historians of all kinds. It is rarely possible to recreate the environment in which a distant event occurred, but it is often possible to infer from scattered relics of the past what exactly happened. Ourisson and Nakatani have cleverly made meaningful relics out of apparently miscellaneous information about the organic content of sediments. What else can be recruited to the same good cause?

John Maddox

Upwardly mobile organics

J. Campbell Scott

THE electrical and optical properties of organic materials provide a rich variety of phenomena for scientific study and technological exploitation. Two of the most thoroughly investigated and commercially successful examples are photoconductors and liquid crystals. On page 141 of this issue¹ a team from the Universities of Bayreuth and Mainz, and from BASF, describe a liquid crystal photoconductor with remarkably high values of charge mobility. Their results demonstrate that the molecular organization of liquid crystals can be used to improve the response time of organic photoconductors.

Since their first introduction in IBM copiers in 1976, organic photoconductors have steadily replaced selenium alloys as the material of choice in laser printers and photocopiers. Their operation depends on the flow of photogenerated charge through a layer of amorphous polymer, into which is dissolved an electrically active small molecule. An electrostatic image is formed on the surface of the photoconductor by first charging it uniformly under a corona, and then exposing to light those areas which are to be discharged. This so-called latent image is then developed by toner, a solid ink consisting of charged particles which are attracted to the desired areas of the photoconductor. The time taken for the latent image to appear, in other words the time between exposure and development, is dictated by the speed with which the carriers move across the transport layer. Charge carrier mobilities are typically in the range of $10^{-5} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ (ref. 2), a value which ultimately limits printing speed and/or size reduction, and which should be compared with mobilities of up to $1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ in organic crystals such as anthracene³. The latter, however, are not useful for printer applications as they do not form large-area homogeneous films.

Liquid crystals were initially used in the early 1970s in watches and calculators. Within a few years, they fulfilled the requirements of low power consumption and flat-profile displays for portable computers, although many might say that the visual quality at that time was barely acceptable. Today, liquid crystals are found in the latest full-colour screens for laptops and on the backs of aeroplane seats. These displays exploit a property which is now popularly termed 'self-assembly' (ref. 4); the molecules align themselves over macroscopic distances leading, in the case of nematic liquid crystals, to optical birefringence in the fluid medium.

The mobility of $0.1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ now reported by Adam *et al.*¹ in the liquid

crystal 2,3,6,7,10,11-hexahexylthiotriphenylene (HHTT) is exciting because it approaches that of anthracene, and yet the material, being fluid, can be prepared as a uniform large-area film. HHTT is a discotic liquid crystal; that is, the molecules are disk-like and assemble face-to-face in columns. In one range of temperature, the columns are ordered (in an average sense, because the material is still fluid) in a hexagonal phase. At slightly lower temperatures, the columns develop a helical twist, and it is in this still-liquid phase that the mobility is highest. Of course, the liquid nature of the material does present containment problems, and precludes its immediate use in photoconductor drums. But one can imagine a processing technique in which the liquid-crystal properties were exploited to spread the material and to align its molecules, followed by a polymerization step to form a rigid film.

The mechanism of charge transport in amorphous organic materials remains an active area of research. It is generally accepted that the motion takes place as a series of hops, in which the charge carrier (electron or hole) resides for a relatively long time on each molecule in its path. This should be contrasted with crystalline materials in which an energy-band description is more appropriate and mobility is weakly temperature dependent, being limited by scattering from impurities and phonons. Hopping mobility is a thermally activated process, but the origin of the activation energy is not yet fully understood. Two processes — energetic disorder and polaron formation — are believed to contribute, but it is not yet clear how to determine the relative importance of each.

Data from the material described by Adam *et al.* may help to shed some light on this issue. The carrier mobility in the supercooled helical phase of HHTT is very weakly temperature dependent (that is, the activation energy is small or perhaps even zero). In this phase, the transport resembles that of organic single crystals. The face-to-face assembly of the disk-like molecules presumably promotes overlap of the π -orbitals and enhances the transfer rate, perhaps even to the point of band formation. There are large and abrupt changes in the mobility at each of the phase transitions as the material becomes progressively more ordered: isotropic, to hexagonal, to helical, to (polycrystalline) solid. One hopes that correlating the structures of HHTT and similar materials with their transport properties will help to clarify the mode of transport and the role of disorder.

Much of the research on the electronic and optical properties of organic materials in the past 30 years has been motivated by the potential for technological application. But the early goals of this research were rather misguided: for example, conducting polymers such as polyacetylene were frequently compared to copper, even though it is unrealistic to expect such polymers to replace copper wiring in either circuit-boards or houses. Similarly, attempts to make diodes and field-effect transistors from semiconducting polymers only reveal their inferiority to silicon. Rather, the successful commercialization of organics has come, and will undoubtedly continue to come, not in applications where they compete directly with well-established inorganic material but where they provide unique properties and processing advantages.

For example, conducting polymers such as polyaniline and polypyrrole are being used increasingly in antistatic coatings and radiofrequency shielding. The conductivity requirements are modest; the advantage lies in the fact that these conductors can be blended with other polymers and moulded into arbitrary shapes without compromising mechanical properties. The continued success of organic photoconductors arises not because they are necessarily better than selenium or amorphous silicon, but because large areas can be coated more cheaply. Another advantage that organics have over selenium is that they are environmentally more benign.

In my opinion, the products that have appeared to date represent only the modest beginnings of organic optoelectronics. Novel properties continue to be discovered and explored in research labs around the world, including, for example, optical nonlinearity⁵, photorefractivity⁶ and electroluminescence⁷. The effort to harness organic self-assembly is equally widespread. It seems inevitable that the most unusual, and therefore most useful, organic devices will result from the marriage of these two subfields. The work of Adam *et al.* represents a major step in that direction. □

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Enter the 'swinging gate'

Sanford Simon

FORTY years ago Hodgkin and Huxley revolutionized biology with a quantitative description of neuronal excitation and a postulate of individual membrane-bound channels for ion transport. Ion channels are now known to be responsible for a plethora of physiologies, such as learning, and pathologies, such as cystic fibrosis. Channel proteins have been purified, their DNA has been sequenced, and the kinetics of ions flowing through individual channels have been analysed. But few tools exist for the structural analysis of membrane proteins and little is known of the conformational changes responsible for channel activity. Now a report on page 158 of this issue by Slatin *et al.*¹ demonstrates that one ion channel, colicin Ia, radically reconfigures itself while opening and closing — and even has a domain that pops in and out of the membrane. The authors show that binding molecules to this 'swinging gate' keeps it from re-entering the membrane, raising the exciting possibility that other ion channels and membrane proteins may have similar gates. Regulation of these channels could ultimately prove to result from different molecules modifying such gates.

Slatin *et al.* investigate whether opening the colicin channel affects those parts of the protein exposed on the surface of the membrane. They achieved this by mutating unique amino acids to cysteine and conjugating them with biotin. After the insertion of colicin into the lipid bilayer, the channels were opened or closed by varying the electric field across the membrane. The water-soluble protein streptavidin, which binds biotin, was then added to the solutions bathing the membrane. Streptavidin on one side of the membrane bound a stretch of amino acids running from residues 511 to 547 when the channel was closed, but not when it was open. Further, binding of streptavidin to these amino acids prevented the channel from opening. Streptavidin added on the opposite side of the membrane did not bind to any of the stretch of amino acids if the channel was closed, but bound a subset of them (511–541) when it was open. Binding streptavidin to these amino acids prevented the channel from fully closing.

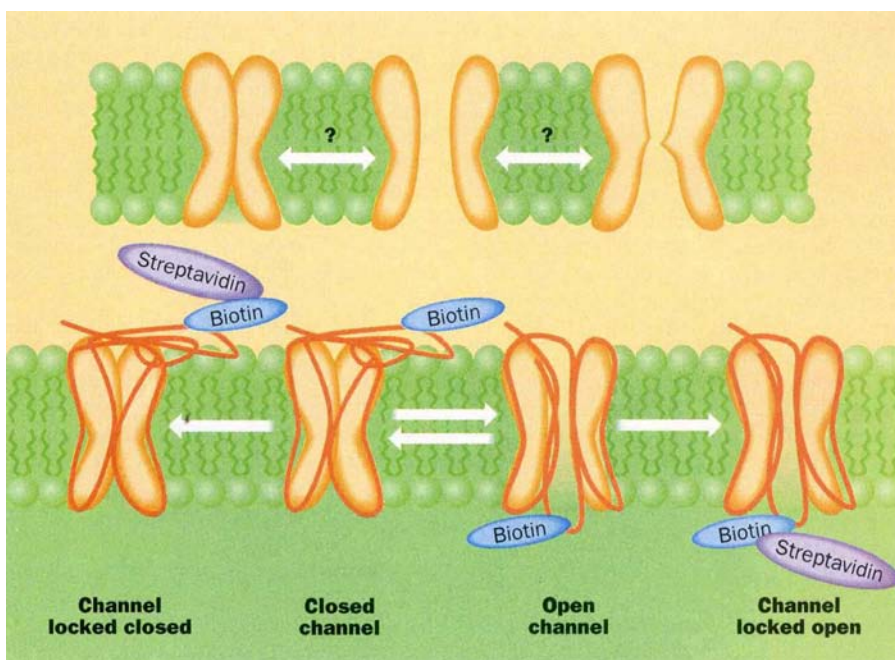
These results are the strongest evidence yet for a major conformational change during the opening and closing of colicin channels. It had been inferred previously from experiments creating new disulphide bonds in colicin to restrict molecular movement² and using chemical labels or protease sensitivity to probe the channel structure³. But what is surprising about the latest results is not only the magnitude of the conformational change but also that

large domains of the protein can reversibly flip across the membrane.

Could other ion channels undergo similar radical changes? Such a molecular model was proposed twenty years ago for the channels of alamethicin and monazomycin⁴. But in the absence of physical evidence for major structural changes or, even more dramatically, domains reversibly dunking through the membrane, it has been generally accepted

channels closing along their entire length after these elegant 'osmotic stress' experiments.

Slatin *et al.*'s most intriguing observation is that at least 31 amino acids can reversibly flip across the membrane. Transmembrane proteins are usually permanently inserted across the membrane during synthesis by the same machinery that is used for secretory proteins⁸. For example, each of opsin's seven transmembrane domains requires the translocation machinery of the endoplasmic reticulum (ER) to be inserted in the membrane⁹. Colicin does not fit this pattern. True, it is a professional membrane perturbant, but



The upper part of the figure shows two models for channel gating. The channel could undergo a subtle shift of structure to block flow through its lumen (right) or it could close down along its entire length (left). The lower part illustrates the principles used by Slatin *et al.*¹ to explore colicin's conformational changes upon channel opening and closing. Unique amino acids on the colicin molecule (solid red line) are mutated to cysteines and then covalently linked to a biotin molecule. Streptavidin added to the upper chamber can only bind to biotin on a stretch of 31 amino acids when the membrane-bound colicin channel is closed. Binding of streptavidin locks the channel in this closed state. Streptavidin in the lower chamber can only bind this biotin-modified stretch when the channel is open, so keeping the channel from closing.

that ion channels undergo only a subtle shift of structure. Most pores are small and a displacement of only a few angstroms should be sufficient to block the conductive pathway. Yet evidence is now accumulating for major structural changes. Chemical reagents can differentially label channels in their open and closed states^{5,6}. Further, the lumens of potassium and mitochondrial porin channels substantially shrink when they close⁷, so each time a channel re-opens it has to extract water from the surrounding medium. Raising the concentration of solutes that cannot permeate the channel increases the energy barrier for channel opening, allowing the internal volume change to be calibrated. Cartoons of channel activity had to be redrawn to show

its behaviour may be applicable to other membrane proteins. For example, the hepatitis-B viral protein is inserted into the ER membrane in a classic signal-sequence-dependent manner, but during subsequent viral assembly a large domain of the protein crosses the bilayer^{10,11}. Although probably all transmembrane proteins require a signal sequence for targeting to and initial translocation into a particular membrane, they may have some domains that can be reversibly embedded in the bilayer. Indeed, for some proteins, like the colicins, certain domains may be continuously flipping in and out of the membrane by thermal energy. This may be particularly relevant for amphipathic domains of ion channels. How this domain crosses the membrane is

unresolved. Is it some property inherent to the swinging gate or to the rest of the colicin molecule? It will be interesting to see whether other domains, even large proteins, can be engineered into this region and flipped across the membrane.

It is not understood how channels such as the potassium channel or cystic fibrosis transmembrane regulator are opened or closed by binding of small molecules or by covalent modification. Let's assume that the physical properties of some domains of the ion channel allow them to bob in and out of the membrane reversibly. Modification of this domain, like the binding of streptavidin to colicin, would then trap the channel in an open or closed configuration. The modification does not induce a conformational change but instead stabilizes changes driven by brownian motion. Thirty years ago it was proposed that brownian motion could drive a machine if given a gradient of thermal (chemical) energy¹². This model may be applicable to ion channels, where thermal motions drive their opening and closing and the chemical energy of modification locks them into one state or another. Such brownian ratchets¹³ have been used to describe protein translocation across membranes¹⁴, filopodial extension and the movement of bacteria and motor molecules¹⁵.

The results of Slatin *et al.*¹ will certainly spur a rethinking among colicin toxicologists and channel physiologists. Together with demonstrations that other hydrophilic molecules such as proteins¹⁶ and nucleotides¹⁷ can also cross membranes through aqueous channels, this discovery should intrigue everyone interested in the functioning of membrane proteins. □

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From the simple to the complex

P. N. Goodfellow and K. Schmitt

THE genes that cause Duchenne muscular dystrophy, Huntington's chorea, adult polycystic kidney disease and cystic fibrosis, to name a few, have all been identified by a combination of classical genetics and molecular biology. These diseases are genetically simple and relatively rare, whereas diseases such as diabetes that impose the largest burdens on Western societies are very common and often genetically complex. Unravelling the aetiology of complex genetic diseases used to be an intractable problem, but recent advances in high-throughput genotyping and high-resolution genetic mapping should lift the gloomy prognosis. As described on pages 130¹ and 161² of this issue, two groups have searched the entire genome to identify new genetic components of a complex genetic form of diabetes known as type 1 insulin-dependent diabetes mellitus (IDDM; see box).

A simple genetic disease can be described by a slight modification of Beadle and Tatum's famous dictum: one gene, one polypeptide, one disease; the inheritance of such diseases follows the laws of mendelian genetics. A complex genetic disease shows no discernible inheritance pattern but has a tendency to cluster in families. Family members share most of their genes but also their environment: both factors can cause familial clustering of a disease — an issue endlessly debated as nature versus nurture. For each disease, the problem can be reduced to how many genes are involved and what, if any, the environmental factors are. The amount of familial clustering of a disease can be quantified by comparing the increased disease risk in a sibling of a patient with the risk in the population as a whole. Comparing the difference in risk between

identical twins and dizygotic twins can give an indication of the genetic contribution to this increased disease risk, leaving the problem of identifying the genes involved and measuring the relative contributions of each gene. For inbred animals and plants, in which both the starting genotypes and phenotypes are well defined, several methods have been successful^{3,4}.

But human genetics demands a different approach. Affected members of a family will share the genes causing the disease and this sharing will manifest itself as an excess of sharing over the 50% expected at random in siblings⁵. Disease genes can be identified by co-segregation with linked genetic markers which will show identity by descent in affected individuals. A genome scan combined with such an analysis makes no *a priori* assumptions about the number of genes involved in a disease, nor the types of interaction between them. The markers used must be close enough that recombination with the disease locus is unlikely and sharing among individuals can only be tested if the markers are polymorphic. The microsatellite markers developed at Génethon and elsewhere are ideal for this purpose: they are highly polymorphic and sufficient numbers of them have been positioned to provide a good coverage of the complete genome⁶.

Nevertheless, the experiments are far from trivial. Hundreds of families containing more than one affected offspring must be found. The more families tested, the smaller is the genetic contribution to the disease that can be revealed. All individuals must be clinically evaluated, their DNA isolated and then tested. A collection of one hundred families requires over 120,000 typings to complete a gene scan of

A brief history of diabetes

DIABETES mellitus was recognized long ago in ancient Egypt but it was not until the last century that investigation began into the biochemical features of the disease, eventually revealing the association between insulin and blood glucose levels that is now well known. There are two types of diabetes, insulin-dependent and non-insulin-dependent diabetes mellitus (IDDM or type 1, and NIDDM or type 2, respectively). IDDM usually affects children and results from an autoimmune response against the pancreas which causes selective destruction of insulin-producing cells and failure of insulin secretion in response to increased blood glucose. As insulin controls the uptake of glucose into cells, this lack of hormone forces them to switch to alternative fuels, with life-threatening metabolic consequences. Lifelong administration of insulin is essential in this condition.

Evidence for the involvement of genetic factors came from animal models (the NOD mouse and BB rat) and the increased risk of developing IDDM for members of a family affected with the disease. Population studies have shown that there is an association of IDDM with certain genes (*DR3* and *DR4*) in the major histocompatibility complex, and other gene associations have now been identified. On the other hand, NIDDM affects adults and is usually due to a combination of impaired insulin secretion and insensitivity of the target tissue to the hormone. These two forms of diabetes affect between 2 and 4 per cent of the UK population.

P.N.G. & K.S.

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large experiment was only possible within the gates of a genome factory; however, adaptation of the microsatellite markers to semi-automated reading on sequencing machines has generalized the technology^{7,8}.

Armed with these new tools, many groups are attempting to analyse the genetic contribution to complex diseases and phenotypes. The results of Davies *et al.*¹ and Hashimoto *et al.*² on IDDM are among the first of the flood. Type 1 diabetes is a good choice for pioneering these methods: not only is it a very important disease but two genes have already been implicated. Reassuringly, the new studies confirm the importance of *IDDM1*, a gene of the major histocompatibility complex (MHC)⁹, and provide more support for a small contribution from *IDDM2*, believed to be the insulin locus itself^{10,11}. Both groups agree on the identification of a new susceptibility locus, in the vicinity of the *FGF3* gene on the long arm of chromosome 11. In a more limited study using a candidate gene approach¹², the same gene association has been detected. It should be stressed that these studies do not imply that *FGF3* is the susceptibility gene; finding this gene and the several others implicated by these studies^{1,2,12} will be the next problem. In simple genetic diseases, *de novo* mutations and gene-inactivating mutations frequently prove the identity between candidate gene loci and disease loci. The gene variants contributing to complex diseases are likely to be common polymorphisms which, in the absence of the other risk factors, do not cause disease. Saturating the region with more polymorphic markers and looking for linkage disequilibrium will help, but genetic approaches will eventually fail because of the complex genetics. Direct tests of the biological effects of the candidate genes will be required to confirm the identity of the susceptibility genes.

One of the most important outcomes of a genome scan is that it can exclude the existence of major contributing genes. The experiments on IDDM rule out other genes with effects as large as the MHC gene and identify several candidate regions for relatively minor genes. In most complex diseases, treatment has been limited to amelioration of the symptoms rather than tackling the underlying

causes. Identification of the new disease-related genes may suggest new therapeutic and preventive strategies and enable environmental influences to be assessed. In the absence of genetic differences predisposing to the disease, environmental differences affecting the development of the disease can be pinpointed, so individuals at risk would have the option of

modifying their exposure to the environmental risk factors. Genome scans may also help to resolve other arguments on nature versus nurture. □

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GEOPHYSICS

Anisotropy and rift systems

Martha Kane Savage

THE theory of plate tectonics, which holds that continents and oceans have been torn apart and huge oceanic plates thrust under other oceans or continents, is well supported and has become a standard principle on which studies of the Earth are based. Yet some of the underpinnings of the theory, such as the relationship between the flow of the asthenosphere and the movement of the overlying lithospheric plates, remain incompletely understood. Numerical models of flow within the asthenosphere have been calculated, but direct observation of the flow is impossible. Measurements of seismic anisotropy, together with assumptions about the relationship between anisotropy and the deformation of the olivine crystals which make up the bulk of the upper mantle, allow us to begin to map deformation of the lithospheric mantle and flow of the underlying asthenosphere. Using the shear-wave splitting technique, S. Gao and others (page 149 of this issue¹) present some of the best evidence to date for the direction of asthenospheric flow beneath a continental rift.

Seismic anisotropy is the property that propagation of seismic waves in one direction through a medium may be faster than that in another direction. Shear-wave splitting results because shear waves have particle motion perpendicular to the direction of wave propagation. When a shear wave enters a simple anisotropic medium, the component that is polarized parallel to the fast axis will begin to lead the orthogonal component, so that the wave becomes 'split' with time. The difference in time between the fast and slow waves is called the delay time, and is proportional both to the length of the anisotropic path and the degree of anisotropy. These measurements yield high-lateral-resolution estimates of the orientation of the fast axis of anisotropy.

Anisotropy in the Earth's crust can be caused by preferred orientation of minerals, or by preferred orientation of cracks². But within the mantle, high overburden pressure and high ductility suggest that few cracks are present, and it is thought that lattice-preferred orientation of the

highly anisotropic olivine mineral is the dominant cause of anisotropy^{3,4}. One response of olivine crystals to strain is that of 'dislocation creep', in which the fast axes begin to point towards the direction of maximum strain. Within flowing material, the maximum strain is parallel to the flow direction. For finite strain, such as that caused by large differential motions between two plate boundaries, the maximum strain is parallel to the plate boundaries⁵. For this reason anisotropy has increasingly been used to study deformation of the lithosphere and the flow of the asthenosphere⁶⁻⁸.

Asthenospheric flow on a worldwide scale has been associated with the 'absolute' motion of the lithosphere above a relatively stable mantle below the asthenosphere through anisotropy determined by global surface-wave inversions⁹. Anisotropy has also been invoked in more detailed studies of asthenospheric flow near oceanic and continental rifts. As Gao *et al.* summarize for oceanic rifts, under the rift axis itself the fast axis of anisotropy may be vertical, whereas on either side of the rift the fast axis is oriented perpendicular to the rifts. Sandvol *et al.*¹⁰ found fast axes along the axis of the Rio Grande (continental) rift to be subparallel to the rift axis. The study by Gao and colleagues is the first to examine anisotropy across a tight array of seismometers laid out in a profile across a continental rift.

The authors have deployed an array of 28 seismometers at 50-kilometre intervals across the 30-million-year-old Baikal rift zone (see Fig. 1 of their paper, page 149). They have come up with a remarkably consistent pattern of shear-wave splitting measurements, in which the orientation of the inferred fast axis is perpendicular to the rift axis. This orientation continues from at least 500 km northwest of the rift, through the rift axis, to about 250 km southwest of the edge of the rift, where the orientation abruptly changes direction. This orientation of the fast axis perpendicular to the rift axis is similar to that observed at mid-ocean rifts. The orientation of the fast axis beyond the abrupt change is consistent with nearby measure-

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ments, and Gao *et al.* suggest that it is caused by Cenozoic deformation of the lithosphere associated with the collision of India and Asia.

The shear-wave splitting technique cannot directly yield the depth at which the anisotropy is present, yet several arguments suggest an asthenospheric source. The abrupt transition in directions at the surface requires the transition at depth to occur in the upper 250 km. The authors further point out that, in the centre of the rift where the lithosphere is thinnest, the magnitude of the signal they receive is too high to be explained by reasonable compositions of material if they are within a thin lithosphere. So they conclude that much of the anisotropy must be attributed to the asthenosphere.

The shear-wave splitting study suggests that the Baikal rift is similar to the oceanic rifts in that the asthenosphere is moving in a direction consistent with movement away from the rift that is being pulled apart. An alternative model, in which the rift is a response to offset in a shear zone, is ruled out because the fast axis of anisotropy does not reflect that expected in response to a shear field.

This first detailed study¹ of asthenospheric flow across a continental rift is an initial step in understanding the relationship between the asthenosphere and lithosphere in continental rifting. Some intriguing questions remain to be explored. The new results suggest that continental rifting may behave like oceanic rifting, in that the sense of motions of the asthenosphere at a distance from the central axis are the same. But how similar are the two phenomena in other respects? Does the anisotropy in the centre of the rift also have a component of vertical motion that might be related to the upward motion of the rifting asthenosphere? Why does the Rio Grande rift exhibit anisotropy that is subparallel rather than perpendicular to the rift direction? Do continental rifts behave similarly in different areas? These questions will be solved only by more careful, detailed studies such as those described by Gao *et al.* □

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Architectural delights

Harry W. Gibson

LITTLE by little, designer molecules are becoming a reality. Molecular boxes, Olympic rings and novel molecular building blocks were just some of the molecular architectures to be unveiled at the recent IUPAC conference in Akron*.

to the surface (E. W. Meijer, Technical Univ. Eindhoven). Among the molecules so imprisoned have been a nitroxyl spin probe, various dyes, the electron acceptor TCNQ and nitrobenzoic acid.

When more than one species is impris-

oned, they can be released selectively. For example, when nitrobenzoic acid and rose bengal are entrapped together, hydrolysis of the t-BOC protecting groups from the amino-acid moieties on the surface liberates the aromatic acid, but retains the dye. Subsequent hydrolytic cleavage of the amino acid from the surface of the dendrimer allows the rose bengal dye to escape. The imprisoned molecules in some cases are tightly constrained and in others less so, causing variations in relaxation dynamics, fluorescence and induced circular dichroism relative to the 'free' molecule. Applications such as imaging processes or controlled release of drugs are conceivable for these molecular boxes.

Interlocked rings — catenanes — have long captured the fancy of artists and scientists, but only recently have such structures bowed to the skills of synthetic chemists. By use of carefully designed components that incorporate the necessary structural information for self-assembly, [4]catenanes² and [5]catenanes have previously been made; one is dubbed

'olympiadane'² from its resemblance to the familiar Olympic symbol. Now a [6]catenane and a [7]catenane (see Fig. 2) have been synthesized (J. F. Stoddart, Univ. Birmingham). These remarkable structures result from molecular recognition between arylene crown ether and bipyridinium-type macrocyclic moieties. The [7]catenane consists of three large interlocked rings with two smaller rings connected with each of the two outer large rings, and produced a molecular ion at a mass-to-charge ratio $m/z=7,130$ in liquid secondary-ion mass spectrometry. Can true 'molecular chains' be far off?

This sort of self-assembly of linear or dendritic systems of controlled size, shape and function is a goal of many research

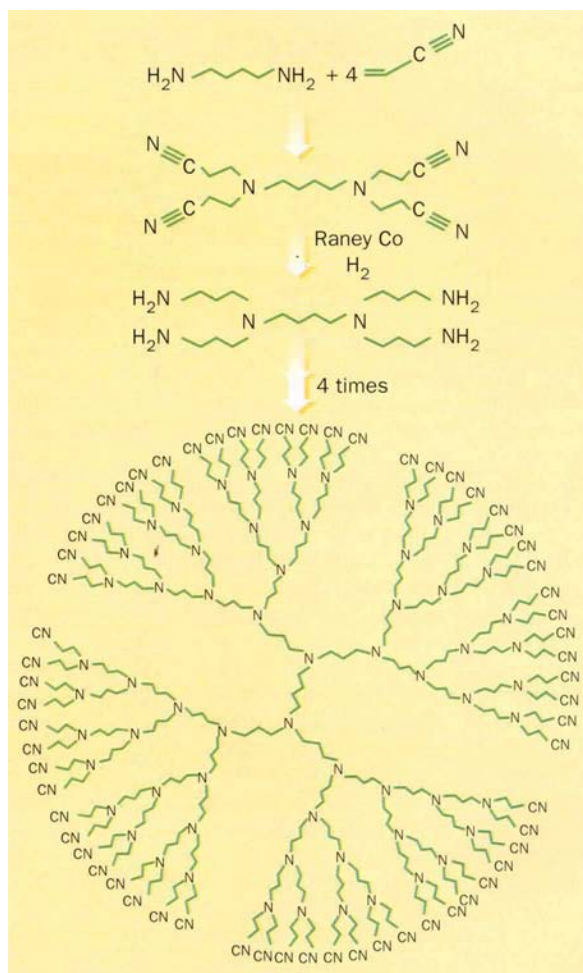


FIG. 1 Synthesis of dendrimer with 64 cyano functional groups; the amino derivative is produced from this by hydrogenation.

It is now possible to entrap molecules of small to medium size in molecular 'boxes' constructed from dendrimers, polymers so branched that they form bushy spheres with sizeable pores between the inner branches. Starting with 1,4-butanediamine and acrylonitrile, dendrimers can be synthesized on large scales¹ (as shown in Fig. 1). Using the higher-generation systems with 32 or 64 surface amino moieties it is possible to entrap molecules by introducing them into the pores and then 'closing the lid' by attaching t-butoxycarbonyl (t-BOC)-protected amino acids, such as phenylalanine,

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*35th IUPAC Symposium on Macromolecules, Akron, Ohio, 11–15 July 1994.

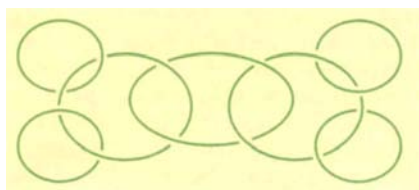


FIG. 2 Representation of the [7]catenane.

groups around the world. Metal complexation of appropriate 2,2',6',2''-terpyridines can achieve this goal — the choice of terpyridines comes about because their fixed arrangement of nitrogen atoms and controlled twisting of the biphenyl type by complexation with various metals lead by self-assembly to helical structures (E. C. Constable, Univ. Basel; see also refs 3, 4). A variety of pyridine-based building blocks can be used to incorporate different functionalities, including photoactivity, electron transfer, charge generation and separation, and liquid crystallinity in helical superstructures. Indeed, by use of quinqué- and hexapyridines, double- and triple-helical strands can be generated for more sophisticated control of the spatial arrangements required for advanced applications such as one-dimensional conductors. And further, by use of well-designed components, dendritic structures incorporating these molecular features have been constructed.

Dendritic polymers have also been shown to be useful initiators for polymerization (J. M. J. Frechet, Cornell Univ.). A dendritic 'wedge' with an alkoxide ion at its apex was used to polymerize caprolactone to the enormous relative molecular mass of $M_r = 310,000$ with a relative molecular mass distribution of 1.07. Small alkoxide initiation of this monomer produces polyester with low relative molecular mass because of 'back-biting' by the active chain end; with the dendritic initiator this is prevented by the steric bulk associated with the wedge. Frechet's group has also attached fullerene molecules to dendrimers⁵ and in Akron reported the preparation of a porphyrin with four dendritic wedges attached, an electron transfer site tucked away in the centre of a globular structure. By allowing controlled access to the molecular centre, this could be useful for chemoselective electron transfer. □

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Heavy isotope record

Arthur N. James

LAST month¹ a team of physicists from Russia and the USA reported the first identifications of mass 265 and 266 isotopes of element 106, by observation of their α -decay mode. Only one other nucleus of mass 266 had previously been identified, which is an isotope of element 109. A nucleus of $^{266}109$ has an odd number (Z) of protons and an odd number (N) of neutrons, each of the odd particles generating a barrier against spontaneous nuclear fission. This kind of barrier does not exist to stabilize the even- N , even- Z nucleus $^{266}106$, so some other physics must inhibit fission.

In the early 1940s uranium was irradiated in intense neutron fluxes and formed many isotopes, increasing the mass number A by neutron capture and the atomic number Z by β -decay. Chemical methods separated minute quantities of new material from old. Some of these radioactive actinide isotopes are now available in milligram quantities. Heavier transactinide isotopes have only been made by heavy ion reactions^{2,3}. The collaboration¹, working in Dubna, Russia, bombarded a few milligrams of $^{248}96$ as target nuclei with 10^{19} ions of ^{22}Ne , each of which had been accelerated to about 10 per cent of the speed of light.

The theory of nuclei is difficult, involving strong forces and strong quantum effects. The key result which theory must match is the terrain formed by plotting contours of nuclear binding energy on a map of N versus Z . Here the naturally occurring nuclei lie along a ridge bounded to either side by shores beyond which nuclei no longer bind nucleons. About 25 years ago Strutinski⁴ revolutionized the techniques used to evaluate the surface of the ridge. The new methods compute detailed quantum corrections (shell effects) to models where the shape of the overall nuclear matter distribution is simply described, principally spherical but including parameters describing departures from sphericity. Common to the families of models since evaluated is their prediction that the ridge descends into a new shore where nuclei are unbound at high A values and that offshore there is almost certainly an island of spherical superheavy nuclei.

Heavy nuclei, although intrinsically unbound, have barriers against disintegration which can be estimated using Strutinski's techniques. By evaluating the consequences of stretching the nucleus, perhaps even letting a waist form, theoreticians may calculate binding energy dependence on shape and find the barriers as different deformation paths are followed towards fission. Certain nuclei in

particular regions of the (N, Z) map become more stable, it seems, as their shape is changed, indicating that deformed nuclei may have long half-lives. There is just such a region^{5,6} between the island of superheavy nuclei and the main ridge, where the shape of the nucleus will resemble a soft drinks can. This prolate deformation with a strong hexadecapole component predicts that the shell correction terms will induce extra stability. The new isotopes $^{265,266}106$ lie near the centre of this region of deformation.

Over the past 30 years much work has gone into developing techniques for manufacturing, isolating and identifying exotic heavy nuclei^{2,3}. After years of preliminary calibrations, the collaborators¹ have produced $^{265,266}106$ nuclei using a heavy-ion fusion evaporation reaction. Ten individual reaction-product nuclei, separated from the neon ions by a gas-filled magnetic spectrometer, are assigned to $Z=106$ isotopes from their specific decay chains. Probably the most extraordinary yet simple observation is that the primary decay mode of these isotopes is α -decay; indeed the high energy of the decays is the main basis for the $Z=106$ identification.

Isotope $^{265}106$ is the most positively identified because one nucleus is followed through three α -decays, where the final two decays show a certain signature that the daughter of the first decay is the known isotope $^{261}104$. Six events are attributed to $^{266}106$. All these have an initial α -decay within 100 keV of 8.64 MeV followed by a short-lived spontaneous fission, a signature indicating an even-even nucleus. The intriguing observation is that the initial even-even nucleus chooses to decay by α -emission. If the α -energy is used to estimate the lifetime of the isotope then a limit can be set on the spontaneous fission rate of $^{266}106$. This last piece of information selects between rival models^{5,6} of how binding energy depends on the shapes through which the nucleus passes before fission.

Nuclear physicists will be content that Strutinski's method has again been vindicated. And the remarkable influence seen here of small shape changes on nuclear properties will undoubtedly stimulate modelling of new nuclear shapes. □

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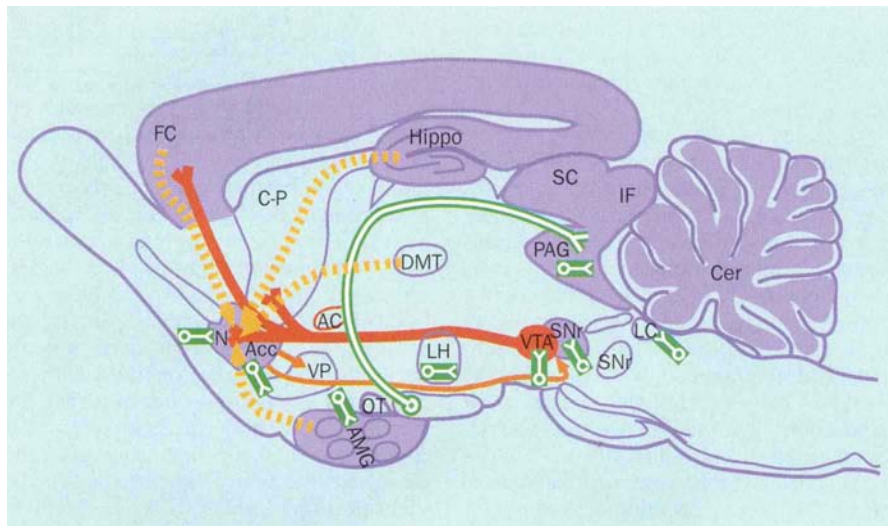
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Dark side of drug dependence

Gery Schulteis and George Koob

ADDICTIVE drugs — such as psychomotor stimulants (cocaine and amphetamines), depressants of the central nervous system (ethanol) and opiate narcotics (heroin and morphine) — in part produce their rewarding effects of euphoria or pleasure through an interaction with the mesolimbic dopamine system. This system involves the dopamine projections from the

ministration of D2 agonists in morphine-dependent rats markedly reduces the severity of somatic withdrawal signs produced by an injection of the opiate antagonist naloxone. These beneficial effects of D2 receptor stimulation are mimicked by localized injections of a D2 receptor agonist into the shell region of the nucleus accumbens. Even more compelling is the



Sagittal rat brain section illustrating several neurochemical systems prominently implicated in the rewarding effects of and dependence on a variety of drugs, including stimulants (such as cocaine, amphetamines), opiate narcotics (such as heroin, morphine), and CNS depressants (ethanol for example). The mesolimbic dopamine system (red) originates in the A10 cell group of the ventral tegmental area (VTA) and projects to the nucleus accumbens (N. Acc.), olfactory tubercle (not shown), frontal cortex (FC), and ventral striatal domains of the caudate-putamen (C-P). Opioid peptide-containing neurons, which may mediate opiate reward, dependence and withdrawal are indicated in green. The opioid peptide systems include the local enkephalin circuits (short segments) and the hypothalamic midbrain β -endorphin circuit (long segment). Yellow indicates limbic system afferents to the nucleus accumbens, and orange represents efferents from the nucleus accumbens thought to be involved in drug reward⁵. Pale blue reflects the distribution of the GABA_A receptor complex which may mediate the effects of CNS depressants such as ethanol. VP, ventral pallidum; LH, lateral hypothalamus; SNr substantia nigra pars reticulata; DMT, dorsomedial thalamus; PAG periaqueductal grey; OT, olfactory tubercle; AC, anterior commissure; LC locus coeruleus; AMG, amygdala; Hippo, hippocampus; Cer, cerebellum. (Figure modified from ref. 2.)

ventral tegmental area in the midbrain to the nucleus accumbens in the basal forebrain¹⁻⁴ (see figure). Some six years ago it was suggested that neural elements in this basal forebrain system may undergo adaptative changes during chronic drug exposure that could drive the development of drug dependence⁵; these adaptive changes may reflect the 'dark' side of drug dependence, that derived from the creation of a state of deficit or need. As shown by G. Harris and G. Aston-Jones on page 155 of this issue⁶, there is now direct evidence that alterations in mesolimbic dopamine function during the development of opiate dependence contribute to the opiate-withdrawal syndrome.

The authors report that stimulation of D2 dopamine receptors by systemic ad-

ministration of D2 receptor antagonists into the nucleus accumbens shell can by itself elicit somatic signs of withdrawal, directly implicating endogenous dopamine systems in the regulation of somatic opiate withdrawal symptoms. To appreciate the implications of these findings, they must be put into the larger context of the current state of knowledge on the neurobiology of drug dependence, and knowledge on opiate dependence in particular.

There is much evidence to support the role of dopamine projections from the ventral tegmental area to the nucleus accumbens in acute opiate reward¹⁻⁴. But there is also evidence that opiates can exert effects in the nucleus accumbens independently of

dopaminergic input from the ventral tegmental area, and that these dopamine-independent mechanisms are sufficient to maintain opiate-reward effects, so both a mesolimbic dopamine-dependent (via opioid receptors in the ventral tegmental area) and a dopamine-independent (via nucleus accumbens opioid receptors) mechanism may operate² (see figure).

Traditionally, there were taken to be two components of drug dependence: psychic dependence, or a compulsion to take a drug to experience its psychic effects and to avoid the affective or emotional discomfort of deprivation; and physical dependence, a state demanding the drug's continued administration in order to avoid intense physical disturbance (somatic withdrawal)⁷. At a theoretical level, this distinction has become increasingly blurred, but has until now been supported by evidence from neurobiological analysis⁸.

As already mentioned, opposing adaptive processes in the primary drug-reward circuitry (that is, the nucleus accumbens) may express themselves during drug withdrawal as affective rather than somatic signs to reinforce compulsive drug use^{5,8}. In contrast, prior work on the neural substrates of somatic withdrawal signs has implicated opioid systems in a number of brain sites in the expression of these symptoms of opiate withdrawal, but not the ventral tegmental area or nucleus accumbens^{1,4,8}. However, Harris and Aston-Jones⁶ have clearly demonstrated that blockade of dopamine function in the nucleus accumbens can elicit certain somatic signs of opiate withdrawal, suggesting that even at the neurobiological level the distinction between psychic and physical dependence may be blurred. In combination with earlier work, the findings of Harris and Aston-Jones therefore suggest that, although the nucleus accumbens may not be involved directly in the elicitation of somatic opiate withdrawal symptoms, this basal forebrain nucleus may have a vital regulatory role, modulating activity in those brain circuits that directly mediate the expression of somatic signs of withdrawal.

There is ample evidence for dopamine/endogenous opioid peptide interactions in the nucleus accumbens and elsewhere in the brain (see figure), and it may be that overstimulation of the opioid peptide system by exogenous opiates leads to decreases in dopamine function^{1,9}. One intriguing possibility, if the dopamine system and opioid peptide systems are considered as independent parts of the motivational circuitry², is that somatic signs of withdrawal are actually a secondary reflection of the affective motivational dysfunction. Indeed, the neural circuitry to support this possibility exists. As discussed by Harris and Aston-Jones⁶, the shell region of the nucleus accumbens directly

projects to limbic (affective/emotional) regions such as the amygdala and lateral hypothalamus, and has direct and indirect (via limbic areas) connections to a number of midbrain and brain stem nuclei which may be involved in the somatic/autonomic signs of opiate withdrawal. Under this hypothetical conceptual framework, continuous activation of the brain's reward circuitry by chronic drug use may lead to neurochemical alterations which, during drug abstinence, result not only in affective motivational deficits, but also in the somatic/autonomic reflection of those deficits.

In this regard it will be important to establish whether currently available laboratory models of affective motivational signs of withdrawal also implicate dopamine systems in the nucleus accumbens in the opiate dependence and withdrawal syndrome. Harris and Aston-Jones⁶ report that preliminary results from one such laboratory model (conditioned place aversion) indicate that a D2 dopamine receptor agonist administered systemically reduces withdrawal aversion; but others have reported that a D2 dopamine receptor antagonist reduces place aversion associated with opiate withdrawal¹⁰. These findings stand in apparent contrast to each other, although both implicate dopamine systems. Further work will be needed to clarify the role of dopamine systems in the nucleus accumbens in the affective motivational signs of opiate withdrawal.

In conclusion, the new findings⁶ indicate that the function of the nucleus accumbens in mediating the effects of drugs of abuse such as opiates extends beyond the acute rewarding (euphoric) effects of these drugs. Furthermore, the direct evidence for a role of mesolimbic dopamine in the withdrawal motivation aspect of drug dependence may prove to be an important key to understanding the neuropharmacological adaptations to chronic drug use which characterize drug dependence. □

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Why testicles are cool

David E. Clapham

LIKE a quarterback with an injured finger, a simple point mutation in the α -subunit of the guanine-nucleotide-binding protein G_s causes the protein to let slip its nucleotide, GDP. The result, as described by Iiri and co-workers on page 164 of this issue¹, is a complex combination of seemingly disparate clinical syndromes. A rare combination in two male patients of precocious masculinization (gonadotropin-independent precocious puberty, or testotoxicosis) and type Ia pseudohypoparathyroidism (PHP-Ia) are explained by a single mutation in $G_{s\alpha}$ which results in a significantly reduced GDP affinity and a temperature-sensitive lability of $G_{s\alpha}$.

In these two cases, testotoxicosis results from the constitutive activation of $G_{s\alpha}$, turning on the enzyme adenylyl cyclase to increase cyclic AMP, so initiating testosterone production in Leydig cells of the testis. This essentially bypasses the luteinizing hormone (LH) receptor, activated normally by the production of the hormone after puberty. In familial male precocious puberty the same outcome results from constitutive activation of the LH receptor² (see table). The interesting twist in this story is that the mutated $G_{s\alpha}$ only survives in the cooler (33 °C) environment of the testis, whereas it is

degraded at the normal body temperature of 37 °C. Thus in other organs, the mutation results in decreased $G_{s\alpha}$ -mediated signalling, which is clinically manifested as a blunted response to both parathyroid hormone and thyroid-stimulating hormone. That there are not more widespread $G_{s\alpha}$ -dependent alterations in other tissues is puzzling, but it may be due to the presence of alternatively spliced $G_{s\alpha}$ heterogeneity of adenylyl cyclase subtypes (of which there are at least eight), and the complex control of the cyclase by $G_{s\alpha}$, $G_{\beta\gamma}$, and calcium ions³. The behaviour of the mutant $G_{s\alpha}$ in various tissues could be determined more definitively in transgenic mice. Survival of $G_{s\alpha}$ might be influenced by association with different $G_{\beta\gamma}$ dimers or by its state of palmitoylation.

Iiri *et al.* demonstrate that the $G_{s\alpha}$ mutant in which an alanine is substituted by serine (A366S) results in an increased dissociation rate for GDP, usually the rate-limiting step catalysed by receptor activation (see figure overleaf). The mutant protein is more rapidly degraded because the increased rate of dissociation of GDP hypothetically increases the time the protein spends in its empty, or nucleotide-free, state. This result is not unexpected: previous work has shown not

Human diseases linked to the G-protein pathway¹⁰

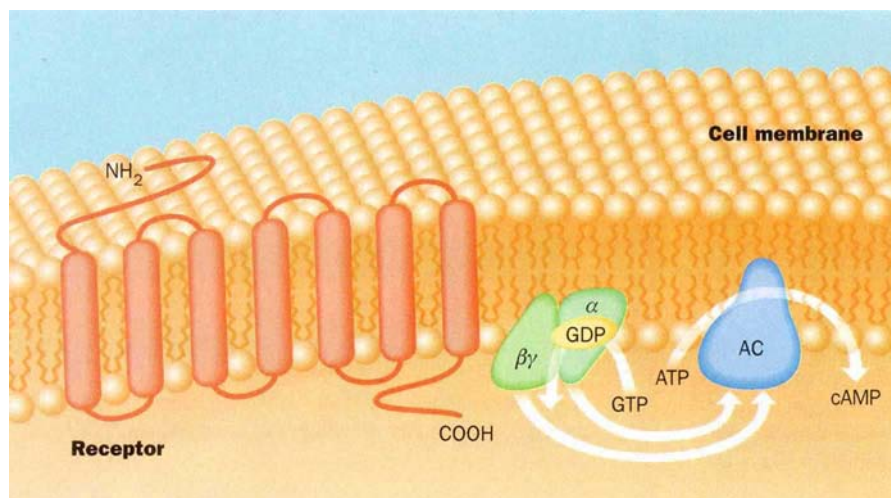
Disease	Defective G protein
Albright's hereditary osteodystrophy and pseudohypoparathyroidisms	$G_{s\alpha}$
McCune-Albright syndrome	$G_{s\alpha}$
Pituitary, thyroid tumours (<i>gsp</i> oncogene)	$G_{s\alpha}$
Adrenocortical, ovarian tumours (<i>gip</i> oncogene)	$G_{i\alpha}$
Combined precocious puberty and pseudohypoparathyroidism Ia	$G_{s\alpha}$

Disease

Defective G-protein receptor

Familial hypocalcaemic hypercalcaemia	Human analogue of BoPCAR1 receptor
Neonatal severe hyperparathyroidism	Human analogue of BoPCAR1 receptor (homozygous)
Hyperthyroidism (thyroid adenomas)	Thyrotropin receptor
Familial male precocious puberty	Luteinizing hormone receptor (LH)
X-linked nephrogenic diabetes insipidus	V2 vasopressin receptor
Retinitis pigmentosa	Rhodopsin receptor
Colour blindness, spectral sensitivity variations	Cone opsin receptor
Familial glucocorticoid deficiency and isolated glucocorticoid deficiency	Adrenocorticotrophic hormone (ACTH) receptor

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Hundreds of G-protein-linked receptors initiate a handful of signal transduction pathways, either controlling intracellular Ca^{2+} , cAMP, cGMP phosphodiesterase, or modulating ion channels¹¹. Bound receptors catalyse the exchange of GDP for GTP on the G_α subunit, presumably dissociating G_α and $\text{G}_{\beta\gamma}$ which then activate enzymes and some ion channels. The G_α -GDP state is stabilized by $\text{G}_{\beta\gamma}$ binding to G_α -GDP and GTP/GDP exchange is the rate-limiting step in the cycle. Iiri *et al.* describe how at the cooler 33 °C of the testis, mutant G_{sa} results in high turnover and increased cAMP levels but in the rest of the body it results in G_{sa} degradation¹. AC, adenylyl cyclase.

only that an adjacent cysteine-to-alanine mutation in the α -subunit of G_α causes the protein to have a reduced affinity for GDP⁴, but also that G_{sa} is most susceptible to denaturation in its empty state^{5,6}. The mutation lies in a highly conserved stretch of amino acids present in all G_α proteins which forms a loop stabilizing the guanine ring of the nucleotide (based on prior mutagenesis and crystal structure data from the α -subunit of another G protein, G_i (ref. 7)). The serine substitution for alanine presumably displaces the guanine ring and disrupts a cooperative network of surrounding hydrogen bonds. One proposal is that receptors catalyse GDP exchange by prying apart the binding cleft between the GTPase structural core and the α -helical cap region^{7,8}.

In a more philosophical vein, Bourne and co-workers¹ offer an alternative way by which GDP might slip from the grasp of G_α : making use once again of the known crystal structures of GTP- and GDP-bound G_α (refs 7, 9), they suggest that GDP leaves at the opposite end of the cleft. The appeal of this idea is that this region of the cleft is where $\text{G}_{\beta\gamma}$, which stabilizes G_α -GDP and blocks G_α -effector interactions, is thought to bind. Perhaps $\text{G}_{\beta\gamma}$ normally tightens the protein's grip to close this potential GDP escape route. Although Iiri *et al.*'s experiments do not address the role of $\text{G}_{\beta\gamma}$ in the mutant protein, and the crystal structure of the $\text{G}_\alpha\text{G}_{\beta\gamma}$ heterotrimer is unknown, one possibility is that the bound receptor catalyses exchange by nudging $\text{G}_{\beta\gamma}$ out of position to allow GDP release from the G_α cleft.

One puzzle in the signal transduction field has been why G_β should be so highly conserved. Hundreds of G-protein-linked

receptors must all initiate the catalysis of GDP/GTP exchange through relatively few G_α subtypes. Perhaps the conformational change in the receptor acts through a common structural $\text{G}_{\beta\gamma}$ interface. Receptor-initiated displacement of $\text{G}_{\beta\gamma}$ (the clasp) would slightly spread the G_α cleft, releasing GDP and allowing GTP to bind, so unleashing activated G_α and $\text{G}_{\beta\gamma}$ to trigger their effects in the cell.

The suggestion of Iiri *et al.* that testotoxicosis be treated by simply warming the testes to body temperature may be oversimplistic. Warming the testes over long periods may irreversibly inhibit spermatogenesis. Furthermore, cryptorchidism, or the failure of a testis to descend into the scrotum during development (so remaining at 37 °C), results in a higher incidence of cancer in the undescended testis. This leads to another interesting possibility, that testicular G_{sa} may participate in tumorigenesis at body, but not testicular, temperatures. □

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DAEDALUS

Fossil rubbish

WESTERN civilization floats on a rising tide of rubbish. Much of it is simply buried at landfill sites, which tend to evolve methane by biodegradation of their organic content. This, of course, is the first step in geological fossilization. In time, buried rubbish should rot down completely to something like coal or oil. Daedalus now plans to help it on its way.

Coalification is a reductive process, inhibited by oxygen but accelerated by heat. Daedalus began to consider reducing hot rubbish with hydrogen; but hydrogen is expensive. He then mused that domestic rubbish contains its own reducing agents in the form of iron and aluminium cans, and that microwaves could quickly make them red hot. Furthermore, microwaves seem to speed up chemical reactions out of all proportion to their heating effect.

So DREADCO's chemical engineers are inventing a microwave-fossilization process for rubbish. They are compacting the stuff in a big cylindrical reaction vessel, essentially a huge microwave oven with a compressive piston. Plastics will melt, paper and food wastes will degrade; metal cans will fuse and burn, removing oxygen from both the included air and the molecules of the rubbish. The resulting carbonaceous residues will soon be conducting enough to absorb microwaves strongly themselves. Thereafter the chemistry should be very fast. It may even evolve enough heat to run spontaneously, minimizing the power required for microwaves. Steam, carbon dioxide and methane will escape, and the material will compact down to its final form.

This form is hard to predict. Under geological fossilization, land vegetation gives coal, and fish and seaweed give oil. Under microwave fossilization, domestic and industrial rubbish (which contains a lot of plastics) might easily give something resembling a low-grade Bakelite, toughened by a filling of metal-oxide particles. Daedalus would be happy with such a structurally useful product, but is equally prepared to accept an oily or coaly output.

When perfected, microwave fossilization should run continuously. Rubbish would be fed into the top of the reactor, and the compacted product would be extracted from the bottom. The evolved gases would be recycled. Steam and methane could be reformed to hydrogen, which could be fed back lower down to complete the reduction process. The output, whether oil or coal or structural plastic, will do its bit to husband our shrinking resources. It may also provide geologists with material for interesting comparisons. David Jones

Climate in the Pleistocene

SIR — White *et al.*¹ proposed a novel method of reconstructing Pleistocene atmospheric CO₂ concentrations based on the stable carbon isotope composition ($\delta^{13}\text{C}$) of mosses and sedges from peat cores. Their technique exploits differences in environmental control over $\delta^{13}\text{C}$ values in tissues of C3 vascular plants and mosses. For mosses, they assume that variation in water content above that required for maximum photosynthesis does not alter $\delta^{13}\text{C}$ values. However, because diffusivity is four orders of magnitude lower in water than in air, external water films create large barriers to carbon assimilation in mosses and fluctuations in water content do significantly influence carbon isotope discrimination.

In the moss genus *Sphagnum*, photosynthetic rates are highly dependent on plant water content. At low water contents, dehydration inhibits photosynthesis, whereas when plants are highly saturated, photosynthesis declines by as much as 50% due to water films^{2–5}. Variation in water content alters internal [CO₂] and should directly influence carbon isotope discrimination Δ .

We used on-line measurements of Δ to assess the consequences of variation in

water content on Δ in *Sphagnum magellanicum* plants (see figure). An analysis of variance test ($n=4$) revealed that water contents significantly alter Δ values (see table; $f>10.5$, $P<0.02$). The mean for the wet plants (at greenhouse water content) was 8.1‰ lower than for the dry plants (blotted dry, but above the optimum for photosynthesis). During on-line measure-

Δ VALUES FOR *S. MAGELLANICUM* PLANTS

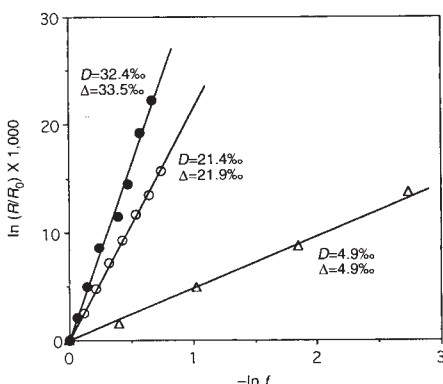
Treatment	Uncorrected (‰)	Corrected for respiration (‰)
	mean $\pm$ s.e.	mean $\pm$ s.e.
Dry	32.9 $\pm$ 1.3	20.7 $\pm$ 0.8
Wet	24.8 $\pm$ 1.7	17.3 $\pm$ 0.7

Respiration affects Δ values in on-line discrimination studies. Corrected and uncorrected results are similar, and show that Δ values are significantly affected by changes in water content ($P<0.02$). Mean discrimination values Δ for wet plants are 3.4–8.1‰ lower than dry ones for corrected and uncorrected data.

ments, however, respiration alters the value of Δ . To estimate the maximum effect of this we assumed that all respired CO₂ measured during dark respiration diffuses into the chamber before refixation. This correction establishes a lower limit for Δ . The corrected mean Δ value is 9.8‰ lower, but the correction factor does not affect the order of means where the wet plants had a 3.4‰ lower Δ value than the dry plants. These results are consistent with field $\delta^{13}\text{C}$ data⁶.

In the field, *Sphagnum* plants experience 4–5-fold variation in water content caused by local effects along hummock-hollow gradients and by seasonal or annual variation in rainfall^{3,4,7,8}. These differences are correlated with >50% variation in measurements of net carbon assimilation rates^{7,8}. Therefore, $\delta^{13}\text{C}$ values in field plant material will be greatly influenced by the local water regime because of variation in water contents. Less negative $\delta^{13}\text{C}$ values will occur in plants grown at higher water potentials.

White *et al.*¹ use variations as small as 5‰ in mosses from peat cores to predict past [CO₂] of the atmosphere. However, such variability could be the result of small changes in local water regimes. Their estimates agree best with [CO₂] measured from the Byrd ice core when $\delta^{13}\text{C}$ values of the mosses change by >5‰ (11,000–13,000 years before present). These large fluctuations are not likely to be due to water content variation. Models that use $\delta^{13}\text{C}$ values from mosses to estimate past climates need to consider the influence of water films on the total resistance to CO₂ uptake and isotope discrimination. To accomplish this, $\delta^{13}\text{C}$ values can be modelled as a linearly decreasing function of



On-line measurements of carbon isotope discrimination Δ . Water films in wet (open circles; 38 g H₂O dry wt⁻¹) plants increase resistance to CO₂ uptake and cause a decline in carbon isotope discrimination Δ when compared with dry plants (closed circles; 23 g H₂O g dry wt⁻¹). The wet ($r^2=1.000$) and dry ($r^2=0.996$) measurements shown are near the mean values for these treatments (see table). The discrimination factor (D) is the slope of the linear regression of $\ln(R/R_0)$ versus $-\ln f$ through the origin⁹, where f is the fraction of the original CO₂ in the reaction chamber and R_0 and R are the isotope ratios (¹³C/¹²C) of the initial and subsequent samples. The discrimination⁹ (Δ) is calculated from D using the equation $\Delta=D/(1-D)$. The relationship shown for maize (open triangles; $r^2=0.999$) estimates a Δ value of 4.9‰, within 0.5‰ of the expected value. Discrimination values are higher than expected for C3 plants ($\Delta=28‰$) because respiration affects chamber f and R . Details of methods are available from the authors.

water content above the optimum water content for photosynthesis and as a constant below it; and water contents can be estimated from vascular plant $\delta^{13}\text{C}$ values as by White *et al.*¹.

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WHITE *ET AL.* REPLY — We are pleased that the change in calibration suggested by Rice and Giles does not affect our reconstruction of the large [CO₂] changes during the last glacial transition. We agree in principle with their argument, but wish to point out that field studies of $\delta^{13}\text{C}$ values in mosses indicate that this effect may not be present in the down-core record at Harbeston. For reasons detailed below, we opted in our Letter¹ for a simpler relationship between water content and photosynthesis, with only a suboptimal effect.

In surveys of surface *Sphagnum* samples from many bogs in Patagonia we have never found a correlation between hummock height and/or hollow depth and $\delta^{13}\text{C}$ of mosses. This is, in fact, one of the first correlations we tried to find, based on the previous studies noted by Rice and Giles. Although the Patagonian bogs we sampled lack very large hummocks, we have sampled relief in excess of 50 centimetres and still found no $\delta^{13}\text{C}$ effect.

In surveys of $\delta^{13}\text{C}$ in living *Sphagnum* in many bogs we find that variability in modern plants can be as large as 3‰ in one bog. Correlations of the $\delta^{13}\text{C}$ changes with water content in the mosses are not significant, although single measurements of water content may not be representative of the average growing season water content. The only correlation has been with distance from the edge of the bog, shown most strongly in small bogs with high surrounding trees. The $\delta^{13}\text{C}$ values get lighter from the centres of the bogs towards the edges, perhaps indicating that mixing in the air may be slowed by the trees acting as wind screens. The isotopically light, respired [CO₂] then changes the atmospheric $\delta^{13}\text{C}$ value, as well as raising the [CO₂] which acts to decrease the effect. Even if there is a masked effect of water content, it is probably less than 3‰. ▶

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Note that much of the surface variability is removed by the temporal averaging accompanying compaction of plants in the core. This is clear from Fig. 1 of our paper¹, which shows little scatter in the down-core $\delta^{13}\text{C}$ record of mosses at Harberton. In this record, each sample represents about one decade. This raises an important point distinguishing down-core studies from process-oriented studies in the laboratory or field. In down-core work, with samples each covering a decade or more, changes in environmental conditions, such as moisture content, must persist to be represented in the average value. Short-term variations are averaged out, and infrequent extremes are poorly represented because of the low assimilation rates during such times. If extremely dry or wet conditions persist, the mosses, or other plants, may not. Bogs can dry up, or become lakes. This is why we have been careful to note species

changes in the Harberton core, and have chosen a bog which appears to have persisted despite the environmental changes of the past 15,000 years.

An assumption in our method is that sedges and mosses record the same moisture conditions, which is to say their relative assimilation rates during different environmental conditions are roughly equal. We are currently checking this assumption by reconstructing moisture levels via pollen assemblages.

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Expansion and emergence of C4 plants

SIR — Morgan *et al.*¹ have provided additional evidence for our hypothesis² that there was a global expansion of C4 ecosystems between 7 and 5 million years (Myr) ago. They point out that their data “do not support previous suggestions of a restricted global date of C4 emergence associated with decreased atmospheric CO_2 around 7 Myr ago (ref. 1)” and that “the emergence of C4 grasses was more complicated than proposed by others (refs 1, 14, 15)” (these refs are cited here as refs 2, 3, and 4 respectively). But Morgan *et al.*¹ misrepresent our conclusions^{2–4}, which addressed the expansion of C4 ecosystems in the late Miocene and Pliocene, not the emergence of C4 plants. The terms are not synonymous, and Morgan *et al.*¹ recognize the distinction in the context of their own record: “In Kenya C4 grasses are present by 15.3 Myr [already emerged] but are not the primary dietary resource of herbivores until 7 Myr [a time of expansion]” (our brackets). Morgan *et al.*¹ argue that C4 plants existed before 7 Myr on the basis of the isotope composition of mammalian tooth enamel, which should enhance the C4 signal above that of the general ecosystem. We agree: in all of our cited papers^{2–4} we found isotope evidence for the existence of C4 plants before 7 Myr ago.

We do not consider that the results presented by Morgan *et al.*¹ contradict our original proposal for rapid expansion of

C4 plants at about 7 Myr. Our work did not address the emergence of C4 plants in general, nor did it propose a restricted date for C4 emergence. Rather, it noted a significant increase in the abundance of C4 plants that was recorded in both the New World and the Old World between 7 and 5 Myr.

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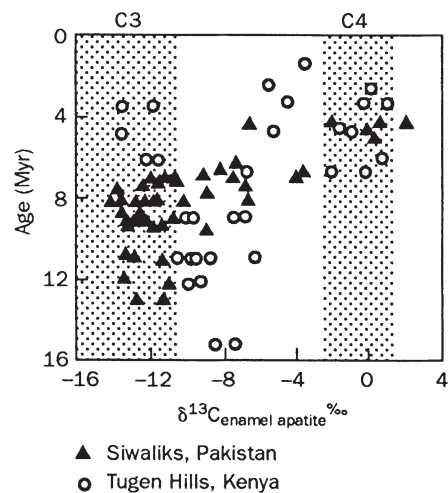
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MORGAN *ET AL.* REPLY — We cannot agree with the claim of Cerling *et al.* that our data¹ support their hypothesis². This hypothesis², that declining atmospheric CO_2 levels drove the rapid global development of C4 ecosystems from ‘virtually’ pure C3 ecosystems, relies primarily on the previously published detailed palaeosol carbonate record from the Siwalik sequence of northern Pakistan, which shows a marked shift from pure C3 to pure C4 vegetation values between 7 and 5 Myr (ref. 3). Other data included in Cerling *et al.*’s paper are $\delta^{13}\text{C}$ values from mammalian tooth enamel². The fossil tooth enamel data from Pakistan include no specimens between about 8.5 and 3.5 Myr and therefore are not relevant. The horse tooth data from western North America

are very limited during the critical time period. Cerling *et al.*² acknowledge that their East African palaeosol data⁴ do not support their late Miocene rapid C4 expansion scenario. Moreover, there are no convincing data to support a change in global CO_2 concentrations for this period.

We addressed the emergence of C4 plants as a notable contribution to mammalian palaeodiet, and by inference palaeovegetation, on two continents, Asia



Values of ^{13}C content ($\delta^{13}\text{C}$) of Siwalik and Tugen Hills herbivore enamel apatite. Stippled areas indicate likely ranges of pure C3 and pure C4 diets. (Modified from Fig. 3 of ref. 1.)

and Africa¹. We used stable carbon isotope ratios of apatite from tooth enamel. Our fossil tooth data from Pakistan¹ (the same local sequences as the palaeosol data^{2,3}) and fossil tooth and palaeosol carbonate data from Kenya^{1,5} do not show an abrupt transition from essentially pure C3 to pure C4 values between 7 and 5 Myr. Fossil tooth data from the two sequences (see figure) show a gradual increase in dietary reliance on C4 plants by grazers and mixed feeders over a period of millions of years beginning 9.4 Myr in Pakistan and 15.3 Myr in Kenya. Palaeosol carbonate data from the Kenyan Rift Valley indicate mixed C3–C4 heterogeneous environments (averaging about 20–30 per cent C4) over the past 15.5 Myr, with no evidence of C4-dominated vegetation at any time⁵.

The data we presented^{1,5} contradict Cerling *et al.*’s² proposal for synchronous global expansion of C4 plants between 7 and 5 Myr from essentially pure C3 ecosystems in response to decreased atmospheric CO_2 levels. We do not dispute that late Miocene global climatic change may have favoured C4 grasses in areas around the world. However, the fossil and palaeosol data from Pakistan and Kenya^{1,3,5} clearly show that the timing and pattern of C4 expansion in Asia and Africa was different. Based on current

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evidence, we maintain that during the middle and late Miocene C4 plants expanded gradually in favourable ecosystems around the world in response to both biotic and abiotic factors.

We do not agree that our use of the word "emergence" caused us to misrepresent the conclusions of Cerling *et al.* regarding the expansion of C4 ecosystems²⁻⁴. We did not intend to interpret our data in the context of the origin or first appearance of C4 plants, and indeed pointed out¹ that we were assessing "the development of modern tropical grassland (C4) biomes".

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No smoking

SIR — Rodu and Cole in Scientific Correspondence¹ report that, by switching to smokeless tobacco, smokers might live 7.8 years longer than by not switching. The authors compare survival rates of smokers, non-smokers and smokeless-tobacco users. The first flaw in this methodology is the assumption that if smokers take up smokeless tobacco, their survival rate will approach that of smokeless-tobacco-only users. No proof is provided for this assumption. The second imperfection is that the amount and length of smoking are not taken into account. Casual smokers will have a very different survival rate from heavy smokers.

Finally, the most disturbing aspect of this study is the conclusion that smokeless tobacco is preferable to smoking on the basis of its only "consequential hazard" being oral cancer. Approximately 30,000 new cases of oral cancer will be diagnosed in the United States in 1994 alone². Of that number, roughly 50 per cent will not survive 5 years from the time of diagnosis. In India, where much of the population uses smokeless tobacco, albeit mixed with leaves of other plants, oral cancer has the highest incidence of any malignancy throughout the body.

In my opinion, Rodin and Cole's advice that health-care providers actively advise patients to switch one addiction for

another is an abdication of our obligation and responsibility to the patient.

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Does diversity beget stability?

SIR — Tilman and Downing¹ present data which they argue support the hypothesis that more diverse plant communities are more stable. This finding, if well founded, would shed light on long-standing questions about the relationship between the diversity and stability of ecological communities²⁻⁴, and provide added motivation to conserve biological diversity. But this finding may be premature.

Tilman and Downing studied grasslands that developed different levels of vascular plant species richness (number of species per 0.3 m²) largely as a result of different amounts of nitrogen fertilization. Fertilization decreases species richness in this system⁵, paralleling a general tendency for grassland diversity to decrease with increasing soil fertility^{6,7}. Community stability in response to drought was measured using the proportional rate of change in total above-ground biomass ($1/B \times dB/dt$), estimated from the logarithm of the ratio of single harvests in successive years. More diverse quadrats showed greater resistance to drought, with less reduction in *B* heading into the drought year 1988; and greater resilience to drought, with faster recovery in *B* after 1988.

As expected from various economic models⁸⁻¹⁰, greater soil fertility generally favours plants with lower allocation to roots versus leaves, higher stomatal con-

ductance, and greater photosynthetic capacity. But plants with low allocation to roots and high stomatal conductance usually have low drought resistance, and plants with high photosynthetic capacity often do. Hence, the fact that quadrats with low species richness had the greatest drop in above-ground biomass during the 1988 drought may be a result of such quadrats having been those most heavily fertilized, and consequently those most likely to be dominated by drought-sensitive plants. The correlation of community resistance to drought and species richness may thus be illusory, reflecting the indirect effects of high soil fertility (in promoting drought-sensitive species or phenotypes) more than the effects of low diversity itself. A similar argument might apply to community resilience, which increased with species richness¹. Plants adapted to low-diversity, high-fertility sites may have been most damaged by drought, and hence the slowest to recover.

Tilman and Downing¹ did attempt to control for possible shifts in the functional groupings of plants caused by shifts in soil fertility along their 'diversity' gradient by examining partial correlations of resistance and resilience with the number and abundance of C4 versus C3 species. But C4 grasses comprise only a small fraction of species richness in Tilman's experimental grasslands⁵, and controlling for their abundance would not screen effectively for systematic shifts in other drought adaptations along the fertility gradient. Furthermore, given that diversity and nitrogen levels are so closely related across plots^{5,10}, it seems highly probable that some within-plot (among-quadrat) variation in diversity is tied to within-plot variation in nitrogen levels and root:shoot ratio; the latter were not measured. Thus, attempts to control for among-plot differences in nitrogen, C3 versus C4 abundance, or 1993 root:shoot ratios (see reply from Tilman *et al.*) are unlikely to eliminate completely the confounding effects of fertility on drought sensitivity.

Some of the variation in species richness among the Tilman-Downing plots reflects a difference in successional age: older fields tend to be more diverse^{5,10}. These older, more diverse fields are known to be dominated by plants with a higher root:shoot ratio than those seen in younger fields^{10,11} (perhaps reflecting more intense competition for nutrients in older fields), which may account for some additional portion of the apparent correlation between stability and diversity.

The underlying problem in the Tilman-Downing study is that diversity, community biomass, soil fertility, and (presumably) species sensitivity to drought are inter-correlated along the experimental gradient. It will require considerable in-

Scientific Correspondence

Scientific Correspondence is a relatively informal section of *Nature* in which matters of general scientific interest, not necessarily those arising from papers appearing in *Nature*, are published. Because there is space to print only a small proportion of the letters received, priority is usually given according to general interest and topicality, to contributions of fewer than 500 words, and to contributions using simple language.

genuity to overcome this problem. One approach would be to select several random subsets including 2, 3, . . . , n species drawn from a large species pool, sow these under a specific set of ecological conditions, allow community assembly (and potential species loss) to occur, and then measure community resistance and resilience. It would be even better to conduct such an experiment at several sites along climatic and edaphic gradients. Only by breaking down the correlations among diversity, community biomass, climate, edaphic conditions and plant adaptations can we determine whether species richness *per se* is an important determinant of community stability in biomass.

There are plausible arguments for either an increase in stability with diversity (such as greater numbers of functionally interchangeable species or species groups, each susceptible to slightly different perturbations; greater segregation of species into compartments that interact little, if at all) or a decrease in stability with diversity (for example, closer packing of competitors along resource spectra, evolution of species-specific mutualisms in diverse tropical habitats, time lags imposed by more trophic levels). It thus seems likely that diversity and stability (particularly with regard to species composition, as opposed to total biomass) may be related positively in some landscapes, and negatively in others.

It may yet be shown that the observed correlation of stability and diversity in the Tilman–Downing study is causal; there are many reasons why, under a fixed set of conditions, assemblages of 2 or 3 grassland species might be less resistant or resilient to disturbance than sets of 15 or 20 species. But even if the Tilman–Downing finding proves to be an artefact of adaptive shifts induced by fertilization and/or succession, it would have no bearing on the fundamental need to conserve biological diversity. Even if more diverse ecosystems were inherently more fragile to perturbation³, the agricultural, medical, scientific, economic and aesthetic values of biological diversity would still make the conservation of native species, communities, and ecosystems one of the world's most urgent priorities.

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TILMAN ET AL. REPLY — There are few long-term ecological studies of biodiversity, and even fewer that encompass a major natural disturbance. Our 12-year study^{1,5,12} of 207 permanent grassland plots thus provides a unique record of the relationships between biodiversity and the stability of ecosystem productivity in response to drought. Givnish suggests that the strong relationship that we observed¹

between ecosystem stability and plant species richness might be an artefact caused by changes in root-to-shoot ratios and photosynthetic physiology associated with nitrogen treatments. Because we had similar concerns about collinear variables when we began our analyses, we used multiple regressions to control statistically for effects of over 20 variables¹. Whether we controlled for all of these simultaneously, one at a time, or in various combinations, there remained in all cases a highly significant effect of plant species richness (biodiversity) on ecosystem stability¹.

Although we did not originally control for root:shoot-ratio shifts, we did measure root:shoot ratios in all plots in 1987 at the start of the drought (RSR₁₉₈₇) and in 1993 (RSR₁₉₉₃), by which time plots had returned to pre-drought biomass and composition. When we include RSR₁₉₈₇, RSR₁₉₉₃ and their interaction (product of RSR₁₉₈₇ and RSR₁₉₉₃) as additional covariates in backwards elimination multiple regressions, we find that part of the dependence of drought resistance on biodiversity is explained by 1993 root:shoot ratios ($F=4.85$, $P=0.03$), as Givnish suggests. The effect is in the right direction, but small (the overall R^2 increases from 0.48 to 0.50). But contrary to Givnish's suggestion, the addition of root:shoot ratios does not eliminate, but rather slightly strengthens, the effect of biodiversity (F increases from 19.25 to 22.53; $P<0.001$ for both). RSR₁₉₈₇ and the interaction term do not significantly contribute to the multiple regression ($F=0.01$, $P=0.92$ and $F=0.25$, $P=0.62$ respectively).

In response to Givnish's concerns, we have performed other analyses that include root mass, changes in root mass, $\log_e(\text{root mass})$, $\log_e(\text{total biomass})$, biomass of additional plant species, and various interaction terms as additional

covariates. In not a single case did the addition of any covariates eliminate the significant residual effect of biodiversity on stability. Thus we doubt if other physiological shifts, as suggested by Givnish, would eliminate the effects of biodiversity on ecosystem stability. Although no single study can eliminate all reasonable doubts about ecosystem functioning, the preponderance of available evidence^{1,13–17} supports the hypothesis that biodiversity influences ecosystem functioning.

We agree with Givnish's proposal that a cleaner test of the effects of biodiversity on ecosystem functioning would come from direct experimental control of species diversity. In 1993 we began such a study. This 23-acre experiment includes 342 large (13 × 13m) and 147 small (3 × 3m) plots that contain 1–32 species randomly drawn from a pool of native prairie plants. These plots are being sampled to determine the effects of plant diversity on plant productivity, insects, plant pathogens, pollinators, and soil carbon and nitrogen.

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C₆₀ complexation revisited

SIR — In the recent paper by Atwood *et al.*¹ on a purification procedure for C₆₀ and C₇₀ by selective complexation with calixarenes, it was implied that we had previously studied the complexation of C₆₀ with cyclodextrins². In fact, we used calix[8]arenes as the selective host species; but ours were water-soluble rather than the water-insoluble variants used by Atwood *et al.*

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Piety in the sky

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The Physics of Immortality: Modern Cosmology, God and the Resurrection of the Dead. By Frank J. Tipler. Doubleday: 1994. Pp. 528. \$24.95. To be published in the United Kingdom by Macmillan early next year.

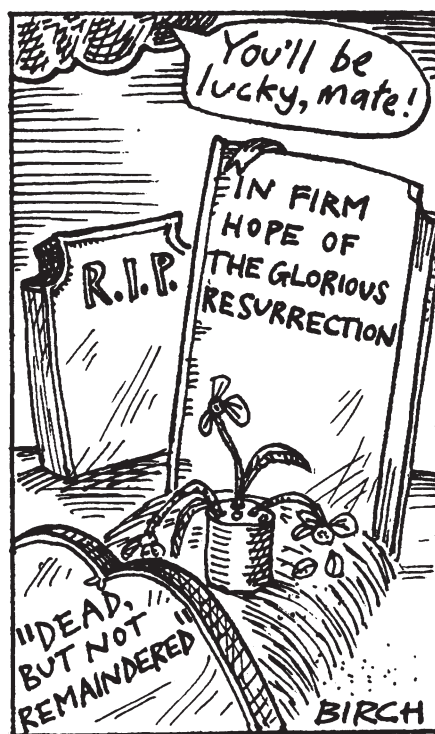
THIS must be one of the most misleading books ever produced. It has a great air of authority and seems highly erudite, covering vast areas of knowledge in a polished way. There are perceptive comments on a wide range of topics throughout. Yet underneath this facade lies wishful fantasy and a total lack of intellectual rigour. It is a masterpiece of pseudoscience.

Frank Tipler deploys his astonishing breadth of knowledge to tackle in an apparently serious manner questions of vital importance. But his claims are fantastic: "This book is a description of the Omega Point Theory, which is a testable physical theory for an omnipresent, omniscient, omnipotent God who will one day in the far future resurrect every single one of us to live forever in an abode which is in all essentials the Judeo-Christian Heaven" (p. 1). All this is accomplished, he claims, on the basis of modern physics alone. He is an ultimate reductionist, who claims to perform one of the most astounding conjuring tricks of all time: his book, he says, shows that "theology is nothing but physical cosmology based on the assumption that life as a whole is immortal. . . Religion is now part of science" (pp. 338–339).

The secret of this trick lies in the consistent misuse of language combined with a blithe disregard for the experimental testability of his completely arbitrary series of assumptions. What one would have assumed was just an undergraduate joke is here presented as if it were a serious theory. But it fails all the criteria for serious consideration, despite being dressed up with spacetime diagrams, causal boundaries, Feynman diagrams, quantum wave packets and so on, presented in many highly technical appendices.

Tipler defines a 'living being' as an entity that codes information, with the information preserved by natural selection. He deduces from this (p. 125) that an automobile is alive — a *reductio ad absurdum* of his entire method of argumentation. His basic assumption is that life continues to exist right until the end of the existence of the Universe, which he further assumes collapses to a singularity in the future (a time-reversed 'big bang'). This ignores the fact that the indefinitely rising temperatures in such a Universe would dissociate not only molecules and atoms but even nuclei into their fundamental constituents, as a result of contin-

ual bombardment by photons at supergiga-electronvolt energies. Both the reliable storage of complex biological information and its systematic and highly controlled hierarchical processing would be completely impossible in these circumstances — yet none of this deters Tipler from his fantasies, which involve total emulation of human beings in a hypothetical computer at an "Omega



Point" (which if it were to exist would actually be outside spacetime, so nothing physical could happen there). This hypothetical process is what he calls "Resurrection". And we are supposed to believe that he has shown scientifically not only that this could happen, but that it necessarily will happen: "If any reader has lost a loved one, or is afraid of death, modern physics says: 'Be comforted, you and they shall live again'" (p. 1).

One cannot point out in a short review all the absurdities in this extraordinary edifice, which is the product of a fertile and creative imagination unhampered by the normal constraints of scientific or philosophical discipline. Tipler does not merely base his theory on highly improbable assumptions and make claims that cannot by any stretch of the imagination

be tested by experiment or observation; he typically assigns the label 'God' to a mathematical construction that, while it might possibly be a good description of the causal boundary of the real Universe (it probably is not), certainly does not correspond in any serious sense to what the word 'God' is normally taken to refer to. He claims to prove — on the basis of physics alone — that this theoretical construction is a loving Being. This is indeed a totally new kind of physics. Out of kindness I refrain from quoting some of his claims about the nature of sex in his hypothetical "Heaven".

The real questions raised by this book are whether the author in fact believes what he has written, and why a respectable publisher has put it out. As regards the first, on page 305 he states: "I do not . . . believe in the Omega Point [because there is not yet any experimental evidence in its favour]". So the whole argument up to here is a game of some kind, an elaborate charade leading hopeful readers on in the naive belief that the author means what he says. For example, on page 1 he writes: "I shall show exactly why this power to resurrect which modern physics allows *will actually exist* in the far future, and why it *will in fact be used*" (my italics). Three hundred pages later he reveals that he himself does not believe the truth of the theory that leads to this statement. There is no reason why anybody else should believe it either: the theory does not and cannot tell us anything about whether resurrection of the dead — in the usual meaning of the phrase — will in fact take place.

On the second point, surely the explanation has to be that the publishers expect to make a lot of money from gullible members of the public who can be taken in by this kind of nonsense. They may be overestimating just how gullible people are. Or could it be that the publishers have fallen for this mirage? Is it perhaps they who are being taken in?

The Physics of Immortality does a considerable disservice to science by making incredible claims for what science can achieve — way beyond what in fact lies within its capabilities. Further, the book will make things much more difficult than before for those engaged in the debate about the relationship between science and theology, a debate that is gaining momentum. One should not make the mistake of believing that this book makes a worthwhile contribution to the discussion. It does not take seriously either science or theology — or indeed its readers. □

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Genetics of a genus

J. R. S. Fincham

Aspergillus: 50 Years On. Edited by S. D. Martinelli and J. R. Kinghorn. Elsevier: 1994. Pp. 851. DFL575, \$328.50.

The Genus *Aspergillus*: From Taxonomy and Genetics to Industrial Applications. Edited by K. A. Powell, A. Renwick and J. F. Peberdy. Plenum: 1994. Pp. 380. \$105.

THE year 1944 saw the publication of a demonstration by Guido Pontecorvo and Alan Gemmell of segregation of genetic markers from heterokaryons in *Penicillium notatum*. They were greatly helped by two convenient features of the fungus — the asexually produced spores (conidia) were uninucleate, and the pigmentation of each spore was determined by its own nucleus. Soon after, Pontecorvo turned to the related fungus *Aspergillus nidulans*, which had the same advantages and the additional benefit of a sexual cycle, and hence the possibility of orthodox genetic analysis.

In the new department of genetics at the University of Glasgow, Pontecorvo, with a small group of young colleagues, worked out methods for handling the new genetic organism. The problem of self-fertility (homothallism) that had initially seemed to make *A. nidulans* unsuitable for conventional genetics was overcome by working out simple methods for selecting 'crossed' fruiting bodies. An unexpected bonus from the work on heterokaryons was the discovery of occasional vegetative nuclear fusion to give hybrid diploids, which were sufficiently stable to keep in culture but had a tendency to segregate genetic markers, both through vegetative haploidization and by diploid mitotic crossing-over. This opened up the possibility, subsequently extensively exploited, of parasexual analysis — genetic mapping independent of the normal sexual process and consequently applicable, in principle, to any of the commercially important but asexual members of the *Aspergillus*/*Penicillium* family.

The major early success of Pontecorvo's Glasgow group lay in the analysis of genetic fine structure. Alan Roper and Bob Pritchard predated Seymour Benzer by a few years in their conclusion that the gene was subdivisible by recombination. The link with biochemistry came after the transplant of the organism, in 1961, to the department of genetics at Cambridge, where Pateman, Cove, Scazzocchio and Arst started grinding it up and assaying for enzymes. *A. nidulans* has since become a favourite model eukaryote in numerous molecular-genetics laboratories around

the world. Though less convenient in many ways than the budding yeast *Saccharomyces cerevisiae*, it is arguably less peculiar — or, at least, it is peculiar in different ways. (I will not be drawn here into the pros and cons of *Aspergillus* versus *Neurospora*.)

Martinelli and Kinghorn have managed to recruit a large proportion of the leading members of the *Aspergillus* community as contributors to their fiftieth-anniversary volume. It is an encyclopaedic compendium, rather tiring to read at 1.7 kilograms, with 24 authoritative chapters backed by full lists of references. Between them, the authors cover almost all aspects of *Aspergillus* genetics, from genetic control of conidiation and sexual fruiting to

The chapter by F. Osman and colleagues on homologous recombination stands virtually alone in addressing classical problems of formal genetics. It does not shed much light on recombinational mechanisms — in fact, *Aspergillus* has contributed rather little in this field — but it does provoke questions. There are at least three remarkable things about *Aspergillus* meiosis. It proceeds without formation of a synaptonemal complex (at any rate, it has been looked for but not found), there appears to be no interference between crossovers, and the frequency of recombination per unit DNA is almost on a par with the ultra-high frequency in budding yeast, which probably holds the eukaryote record in this respect.

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Fruiting bodies of the fungus *Aspergillus niger* (SEM; magnification, $\sim \times 100$).

the mitochondrial genome, gene cloning and heterologous gene expression.

The theme that comes over most strongly is the continued effectiveness of using mutants, selected for their phenotypic effects without prior knowledge of their molecular basis, to uncover systems of control of metabolism and development. Most of the regulatory systems described have been in the literature for a decade or more, but are now becoming increasingly well characterized at the DNA level. They all tend to complexity, with networks of interactions rather than linear chains of command.

Particularly remarkable is the number of examples in *Aspergillus* of clustering of functionally related genes. The genes in each cluster, though subject to some degree of common transcriptional regulation, are separately transcribed, and it is not clear what significance the clustering has. The trend is towards explanations in terms of shared transcriptional enhancers and/or local control of chromatin structure, but mutants with cluster-wide effects that might lead to tests of these possibilities are not easy to find.

Are these features in any way connected?

As one might expect in a commemorative volume, there is a certain amount of personal reminiscence and history, including a foreword by Pontecorvo. Martinelli contributes an interesting summary of how the *Aspergillus* community grew and where everyone came from. Less happily, she uses her editorial prerogative to couch the whole piece in pseudo-biblical language, with Pontecorvo represented as the founding deity. I must say that, extended over three pages, the device wears a little thin. And the statement that Pontecorvo "begat" the distinguished biometrical geneticist John Jinks surely falls short of gospel truth, and may well cause some raised eyebrows in Birmingham.

The second *Aspergillus* book comes from a conference, held under the aegis of the Federation of European Microbiological Societies in 1993. Several of its 28 contributions cover the same ground as chapters in Martinelli and Kinghorn's compendium, but the greater part of the volume is about the role of *Aspergillus* species (not *A. nidulans* in particular) in the world beyond the laboratory. For

many, the main importance of *Aspergillus* is as a source of toxins — aflatoxins and others. Several contributors cover the chemistry and biosynthesis of the toxins and their various impacts on agriculture. *Aspergillus* is also industrially important, and the volume also includes accounts of its use in production of organic acids and extracellular enzymes and in fermentations used in the food industry. Some attention is also given to *Aspergillus* as an occasional human pathogen. This conference book is likely to be more useful and less ephemeral than most in that it brings together information that is otherwise rather widely dispersed. □

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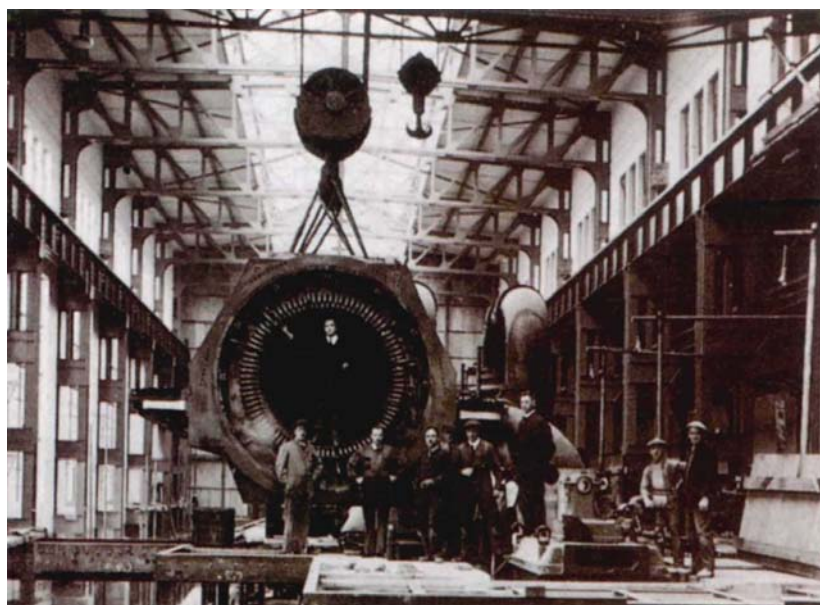
Green business

David Pearce

The Ecology of Commerce: How Business Can Save The Planet. By Paul Hawken. Weidenfeld and Nicolson: 1993. Pp. 250. £17.99.

A SURE sign that an idea has come of age is when it is borrowed, repackaged and sold as new. In *The Ecology of Commerce* Paul Hawken has done exactly that. The message is that we do not pay the full cost of production of the goods and services we buy. Those goods and services use up scarce environmental resources — the global atmosphere, the oceans, the air we breathe, the landscapes we admire — but usually only part, and often none, of those environmental costs is reflected in the price in the market-place. By treating the environment as a free good, we have degraded it, only to find that it is not free after all and that we are suffering from this fundamental economic folly. The answer then is to put the value of environmental resources into production costs and market prices. The mechanisms for doing this are many and varied. Pollution taxes and charges are one way. Pollution permits that can be traded in special markets are another. Deposit-refund systems (the old 'penny on a bottle') are yet another. The more polluting the product the more it will cost, sending a signal to producers to switch to cleaner technology, and to consumers to switch to cleaner products. Then, and only then, can it be left to the market-place to settle what and how much gets produced.

If it sounds familiar, it is. Hawken acknowledges his intellectual debt to Arthur Pigou who held the chair of political economy in Cambridge, England, for 35 years from 1908 (though, curiously, he re-christens him Nicholas Pigou). What



IRONBRIDGE in Shropshire, England, was at the forefront of developments in coal, iron and steam. But it was a late arrival to the brighter world of electricity. By the late 1930s however, when most of the ironworks and coalmines had closed, the region had become a focus for both the generation and distribution of electricity across Britain. This photograph shows Ironbridge "A" Power Station under construction in the years around 1930. The neighbouring "B" Station, still in operation today, was built in response to the demands of the boom years of the 1960s. The picture is taken from *Ironbridge and the Electric Revolution* by Michael Stratton (John Murray, £9.99), a slender well-illustrated paperback that will appeal to all with an interest in British social, economic, industrial or architectural history.

might have been called Pigovian economics is today known as welfare economics, benefit-cost analysis, even environmental economics. And that has been popularized by many economists.

Why then has Hawken attracted such attention for repeating what others have done? First, he probably says it all more persuasively. There are a few academic references in the book: source material is mainly newspaper and other popular articles. Second, he is a businessman, and a successful one. As such he speaks for, as well as to, business. He is right to say that nothing much will change until business changes. The reality is that businessmen and women have the power to change things, and that any attempt to change the world against the "natural impulses" of human beings, as he puts it, will very probably fail. Third, but unfortunately, Hawken clearly believes in some kind of ecological collapse if things are not changed rapidly, and so he latches on to some disastrous examples of the way to go. As just one example, he believes the German 'Duales' system of recycling waste is to be applauded. The system obliges producers of packaging to take the packaging back unless they can guarantee to recycle it to target levels set by the government. Never mind that the result has been a flood of useless materials, depressing prices and encouraging waste to be dumped abroad, to the detriment of

recycling schemes elsewhere. It is surprising to see this scheme lauded in the same breath as ecological taxes: they are two very different things. The Duales system is a muddled mix of eco-taxes and old-fashioned command and control, all backed up by a threat that failure to recycle will result in something worse — 'greenmail' rather than blackmail. As to ecological collapse — it could happen but it is doubtful. The likelihood is simply that of a far more unpleasant, far more insensitive, but wholly sustainable world.

But no one can doubt Hawken's sincerity. The idea of business mimicking nature, not exceeding the capacity of the Earth to absorb wastes or depleting its overall natural stocks, is appealing, if once again familiar. The sustainable business, as he puts it, engages in production processes that are "human, worthy, dignified and intrinsically satisfying". The idea of a new world trade order that rewards those who practise sustainable resource use with low or zero tariffs is also attractive. If Hawken can say all this, if he can make others believe this is a truly fresh look at business and the environment, and if he can have real influence, then all power to his environmental elbow. □

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Mock turtles — the green sea turtle (*Chelonia mydas*) has been dubbed “the world’s most valuable reptile” and “the buffalo of the sea”. These hatchlings were bred at the Cayman Turtle Farm in the British West Indies, a commercial venture that began as Mariculture Ltd in 1968. The story of the farm is told in *Last Chance Lost?* by Peggy D. Fosdick and Sam Fosdick, who capture the entrepreneurial spirit of biologists faced with restrictive legislations, dedicated adversaries and limited capital. It is also a dispassionate and balanced look at the complex controversy surrounding the sustainable use of threatened or endangered species. Published by Irvin S. Naylor, 100 Boxwood Lane, York, Pennsylvania 17402, USA at \$49.95.

Separate estates

Kenneth F. Kiple

Science and the Practice of Medicine in the Nineteenth Century. By W. F. Bynum. Cambridge University Press: 1994. Pp. 283. £40, \$54.95 (hbk); £12.95, \$15.95 (pbk).

DESPITE the challenges still posed by chronic ailments such as cancer and epidemic diseases such as AIDS, modern medicine has accomplished much, gaining in the past few decades an air of self-confidence. Many of its practitioners may therefore be startled by the message of this book: what we regard as modern medicine, the author argues, in fact rests solidly on nineteenth-century foundations.

Between 1800 and the Second World War, science (or at least its claims) increasingly provided the basis for much of medicine. Bynum begins by retreating to 1790 to examine the medical professions in England, France and, to a lesser extent, northern Europe, along with the education of those who staffed them, the luminaries of the day and their state of knowledge (including the classification of dis-

ease). The emergence of hospital medicine following the Enlightenment inspired some of these institutions to house the dregs of society as well as treat the ill. In cities such as Paris, London, Vienna and Philadelphia, the hospitals also became places for medical teaching and research. Bynum analyses the French model for these activities, focusing on pathological anatomy, disease diagnosis and the development of a numerical approach to disease and therapeutics.

Community medicine and disease prevention soon also grew in importance because of the urbanization that accompanied industrialization. We meet several of the leading reformers of the period (and their programmes), such as Johann Peter Frank and his proposed system of medical police, which made an impact in Eastern Europe and Germany; John Haygarth’s ‘fever’ hospitals in the industrial cities of England; and, of course, Edwin Chadwick, whose 1842 report urged that the weapon of cleanliness be aimed at the miasma spawned by filth.

Lemuel Shattuck in Massachusetts, Louis René Villermé in Paris and Rudolf Virchow in Germany are also briefly mentioned, as are some of the dramatic breakthroughs in preventive

medicine, such as John Snow’s elegant epidemiological investigations of 1854 that implicated the Broad Street pump in the spread of cholera in London. The importance of smallpox vaccination was brought home during the Franco-Prussian War: vaccination of German soldiers but not the French meant that only 300 of the former succumbed to the disease as opposed to more than 20,000 of the latter.

The model for laboratory medicine, which Bynum examines next, was German, with the French understandably less enthusiastic than the British, and the German influence most apparent in the United States. But by the end of the nineteenth century “scientists had established themselves, with varying degrees of success, as a kind of separate estate within medicine”. Pulling together threads spun under the headings of chemistry, microscopy, bacteriology and antiseptic surgery, Bynum then demonstrates the extent to which “scientific disciplines were basic to disease formulations by the century’s end”. The rivalry between Koch and German bacteriologists on the one hand, and Pasteur and his French followers on the other, is introduced here, and continued in the chapter “Medical science goes public”, which touches on the triumphs of tropical medicine, the great research institutions, immunology, the pharmaceutical industry, experimental physiology and the discovery of X-rays.

Readers are then treated to an especially fascinating chapter on doctors and patients, which traces the development during the nineteenth century of the medical profession in medical schools and hospitals, with accounts of the emergence of modern nursing and greater public acceptance of the medical profession. The period was marked by the trend toward specialization and struggles over the issues of national health insurance and women doctors. Finally, the experience of being a patient is explored in the poignant story of Alice James, sister of Henry and William James, a ‘career patient’ whose ailments, real and imagined, kept her under the care of doctors for most of her short life.

So did science matter? Although medicine is probably entitled to little credit for declining mortality in the nineteenth century, the support given to scientific medicine by the state conferred status, prestige and credibility on its practitioners, even on doctors who had little interest in science. All of this helped doctors enormously in their drive to monopolize health care.

This is a very fine and important book that will doubtless be judged as definitive. It is wonderfully written, well organized and witty, and rests on impeccable scholarship resulting from an exhaustive examination of the literature. □

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Potassium channels and their evolving gates

Lily Yeh Jan & Yuh Nung Jan

Potassium channels allow potassium ions to flow across the membrane and play a key role in maintaining membrane potential. Recent research has begun to reveal how these channels transport potassium in preference to other ions, how their activity is controlled, and how they are related to other channels.

A CELL'S membrane potential, the difference between the electrical potential inside and outside the cell, is partly determined by potassium channels and the $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ ¹. The $(\text{Na}^+/\text{K}^+)\text{-ATPase}$ helps maintain an asymmetric distribution of potassium ions with a higher potassium concentration inside the cell.

Potassium channels regulate a wide range of cellular functions, including salt and water flow from kidney cells² and guard cells³, insulin release from pancreatic β -cells, electrical excitability and synaptic plasticity in neurons, and even the rate at which the heart beats¹. Recent molecular, biophysical, biochemical and electrophysiological studies have shown how intercellular signalling molecules, cytoplasmic factors and changes in membrane potential modulate the activity of these channels. We now know how they discriminate between potassium and other ions without compromising their efficiency, and can begin to consider their evolutionary relationship with other channels.

Potassium channels of known sequence fall into two distantly related families, the voltage-gated and inwardly rectifying channels. The activity of voltage-gated channels, which have six putative membrane-spanning domains in each subunit, is intrinsically sensitive to changes in the membrane potential¹. These channels are typically activated at membrane potentials above the resting potential (the potential that causes no net flow of ions across the membrane, typically around -60 mV). In animals, this is slightly above the potassium equilibrium potential¹, but in plants, it is slightly below it³. Inwardly rectifying potassium channels⁴⁻⁷, by contrast, have only two putative transmembrane spanning domains in each subunit and are sensitive to changes in the potassium concentration. They are effectively unidirectional, however, in that they allow a much larger potassium influx than efflux, allowing them to control the resting potential without causing massive potassium loss¹.

Potassium-selective ion flow. All potassium channels are at least 100 times more permeable to potassium ions than sodium ions, and allow greater than 10^6 potassium ions to pass per second¹. How can the channel pore interact with potassium so as to maintain this degree of discrimination in an interaction lasting less than a microsecond? Since sodium has a smaller ionic radius than potassium, it is unlikely that selectivity is achieved by physical occlusion.

Several lines of evidence indicate that each channel has a long pore with multiple binding sites arranged in a single file¹. As potassium concentrations increase, the current-voltage relationship deviates from predictions based on the assumption that the passage of one ion through the pore is independent of other ions. Coupled diffusion of multiple potassium ions through the pore has also been demonstrated by tracer measurements of potassium efflux and influx, and more than one blocking ion (for example caesium) has been shown to occupy a pore. Moreover, by mixing two different types of permeant ions (for example potassium and thallium), one can show that the permeant ion with higher affinity for the binding sites (and longer

residence time) slows the passage of the other ion type. Ion flow is therefore less efficient than when the channel is exposed to either of the ions alone, a phenomenon known as the "anomalous mole fraction" effect. Finally, the existence of multiple binding sites has been confirmed by measuring the affinities of three potassium binding sites and a barium binding site in a calcium-activated potassium channel⁸. When these sites are occupied by potassium, the resulting electrostatic repulsion would facilitate ion passage through the pore¹.

The pore. Mutagenesis has been used to confirm the presence of multiple potassium binding sites and identify structural elements involved in ion flow. Voltage-gated potassium channels contain four α -subunits, each with six hydrophobic segments (S1-S6) and an H5 segment between S5 and S6 (Fig. 1), and four hydrophilic β -subunits, which probably associate with the α -subunits on the cytoplasmic side of the membrane^{1,9,10}. Numerous subtypes of both the α - and β -subunits with subtly different properties exist. Initial studies¹¹⁻¹³ may have encouraged the oversimplified view that the H5 segment is the pore-forming structure, but more recent work has shown that mutations in S6 and the S4-S5 loop also affect potassium permeation and/or sensitivity to blocking ions¹⁴⁻¹⁶.

The S4-S5 loop and the H5 segment are thought to be located near the ends of the pore, because they interact with specific pore-blocking peptides applied from the intracellular and the extracellular side of the membrane, respectively^{14,17}. One might imagine that the S4-S5 loop and the H5 segment (and perhaps sequences immediately following S6) fold into the pore, contributing to the formation of constrictions or potassium binding sites (Fig. 1). A role for 'loops' (sequences connecting putative membrane spanning segments) in binding solute for facilitated diffusion across the membrane seems fairly general. The substrate affinity of the sugar-specific channel in the bacterial phosphoenolpyruvate-dependent phosphotransferase (PTS) system is affected by mutations in loop residues¹⁸, as is the potassium affinity of the bacterial high-affinity potassium transport protein KdpA¹⁹. Moreover, structural analysis of the bacterial outer membrane channel porin has shown that a loop folds into the pore, determining the ion selectivity of the channel²⁰.

In the α -subunit of voltage-gated channels, all of the structural elements known to be involved in potassium permeation reside in the C-terminal half of the hydrophobic core¹¹⁻¹⁷. The subunits of inwardly rectifying channels contain only two hydrophobic segments, M1 and M2, separated by an H5 segment⁴⁻⁷. As M1, M2 and H5 show sequence similarity with the S5, S6 and H5 segments of voltage-gated channels^{4,5}, it appears that the two classes of potassium channels share the same basic pore design. Whether this design reflects the pore structure of an ancestral channel will be discussed later.

Multiple gates for voltage-gated potassium channels. Mechanisms that control channel activities are referred to as "channel gating", and the molecular entities involved in gating are termed "gates". The activity of voltage-gated channels is

controlled by at least 2 gates: a voltage sensor, which controls when the channel opens, and an inactivation gate, which controls the duration of channel opening¹ (Fig. 1). These channels are intrinsically sensitive to membrane potential, which generates an electrical field in the hydrophobic membrane interior. They must therefore have membrane-embedded charges or dipoles which can monitor changes in this field and induce an altered conformation leading to channel opening. Mutagenesis studies indicate that the S4 sequence, which contains a mixture of basic and hydrophobic residues, forms part of the voltage sensor^{21–23}.

Inactivation of the channel can be partially accounted for by the ball-and-chain model²⁴: a cytoplasmic gate intrinsic to the channel ('the ball') binds to the cytoplasmic mouth of the pore ('the receptor') after the channel opens, thereby occluding the pore. This model was initially suggested by the observation that limited exposure of sodium channels to cytoplasmic proteases selectively abolished channel inactivation. In voltage-gated potassium channels, the ball corresponds roughly to the first 20 amino acids of its α - or β -subunit and is still functional in the form of a peptide in solution^{25,26}, indicating that covalent linkage is not necessary for inactivation gating.

The involvement of a domain found in both the α and β subunits suggests that the wide range of kinetic properties observed in potassium channels *in vivo* may arise from the mix and match of different α - and β -subunit subtypes. The receptor for the ball probably contains residues in the S4–S5 loop near the cytoplasmic end of the pore¹⁴. Another inactivation mechanism, independent of the ball-and-chain machinery, involves the H5 and S6 segments and is sensitive to the type of permeant ions present²⁷.

Permeation-coupled gating of inwardly rectifying potassium channels. Inwardly rectifying potassium channels conduct potassium ions better at membrane potential below the potassium equilibrium potential, allowing a preferential flow of potassium from the extracellular to the intracellular space¹. When external potassium concentration is altered, the potassium equilibrium potential changes according to the Nernst equation and the voltage-dependence of these channels changes accordingly, so as to preserve inward rectification. The gating of inwardly rectifying potassium channels is therefore tightly coupled to the electrochemical force driving the potassium ions.

These properties are partly a result of the ability of an intracellular (or cytoplasmic) magnesium ion to enter the pore and block ion flow^{28,29}. The magnesium ion can not traverse the pore, but can be displaced by incoming potassium, leading to inward rectification. The situation thus resembles that seen in the NMDA (*N*-methyl-D-aspartate) receptor, in which a voltage-dependent block by external magnesium ensures that the channel only conducts when it has bound glutamate in the presence of a raised membrane potential³⁰. The difference in magnesium sensitivity between the inwardly rectifying potassium channels IRK1 and ROMK1 has been attributed to a difference in their C-terminal hydrophilic domain³¹.

Inwardly rectifying channels also contain an intrinsic gate, however, which ensures inward rectification even upon removal of cytoplasmic magnesium³². This gate is influenced not only by the electrochemical force driving the potassium ions, but also by the magnesium ion in the pore; the closing of the channel by the intrinsic gate is slowed when the magnesium block is present³². Whether this coupling between ions in the pore and

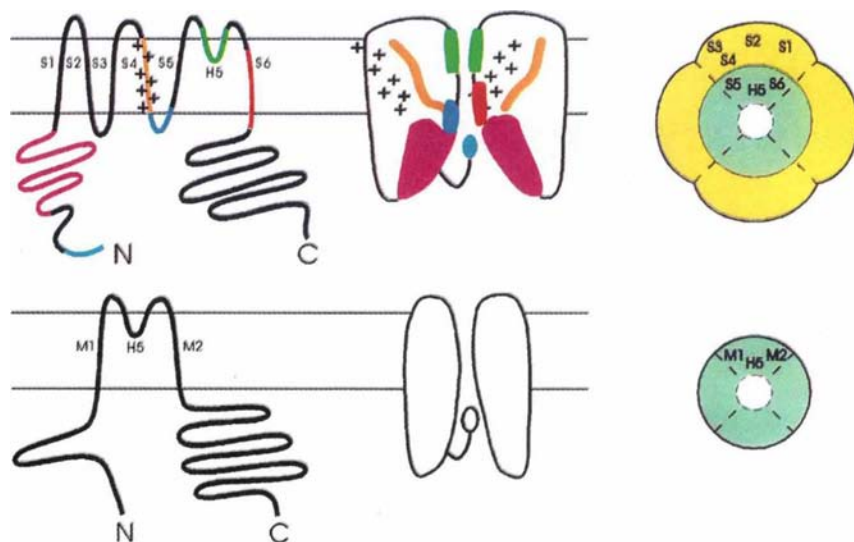


FIG. 1 Proposed transmembrane topology (left) and model (side view, centre; top view, right) of voltage-gated potassium channels (upper panel) and inwardly rectifying potassium channels (lower panel). The S4 sequence contains basic residues and may be the voltage sensor^{21–23}. The N terminus of the Shaker voltage-gated potassium channel α -subunit corresponds to the cytoplasmic inactivation gate (the ball)²⁵, which interacts with the S4–S5 loop¹⁴ and occludes the pore. The S4–S5 loop, H5 and S6 segments are involved in ion permeation^{11–17}. The highly conserved N-terminal hydrophilic domain as well as S1 contribute to α -subunit interactions^{48,49}. Voltage-gated potassium channels contain four β -subunits in addition to the four α -subunits shown in this figure¹⁰. Whether inwardly rectifying potassium channels also contain β -subunits is not known.

the intrinsic gate reflects a close physical association between the pore and the gate is an open question.

The activity of various members of this family is controlled by other ligands, a process that may involve additional gating machinery. Limited cytoplasmic proteolysis of ATP-sensitive potassium channels increases their activity and reduces the inhibitory effect of ATP³³, while similar treatment of muscarinic potassium channels renders them constitutively active in the absence of G proteins³⁴. These results indicate that these channels may be closed by a cytoplasmic gate, analogous to the ball-and-chain inactivation gate of voltage-gated channels. This closed state is apparently stabilized when ATP binds ATP-sensitive potassium channels or when activated G-protein subunits dissociate from muscarinic potassium channels.

The cytoplasmic gate as an universal gating mechanism. It is now clear that cytoplasmic gates control the activity of chloride channels as well as potassium channels and voltage-gated sodium channels. Known chloride channels fall into two types, the CIC family (each of whose members has 12 putative membrane-spanning segments)³⁵, and a member of the ATP-binding cassette (ABC) superfamily, the CFTR (cystic fibrosis transmembrane regulator) channel affected in cystic fibrosis, which contains two domains each with 6 hydrophobic segments and an ATP-binding cassette, separated by the so-called R domain³⁶. Sequences in the hydrophilic N-terminal domain of the ubiquitous CIC-2 chloride channel allow this channel to be activated upon cell swelling. Transplanting these sequences to the C-terminal hydrophilic domain does not impair the gating function, suggesting that cell volume regulates this channel with a ball-and-chain gating mechanism³⁵. Activation of the CFTR chloride channel apparently requires conformational changes associated with ATP hydrolysis³⁷ and phosphorylation of the R domain, which probably functions as a cytoplasmic gate, as the channel becomes constitutively active when it is deleted³⁶.

The channel need not be covalently linked to the cytoplasmic gate. Certain bacterial PTS gene complexes, for example, contain separate genes encoding the phosphorylated domain and the sugar-specific channel¹⁸. Similarly, the ATPase activity and the transport activity of both the *E. coli* Kdp potassium transport

system and certain members of the ABC superfamily (such as the bacterial histidine transporter) are encoded by separate genes^{18,19}. Moreover, activity of the *Drosophila* potassium channel Slowpoke (a calcium-activated member of the voltage-gated family) is modulated by cytoplasmic calcium even when the calcium-sensitive C-terminal domain is expressed as a separate protein³⁸. Covalent linkage thus appears to be dispensable for the protein-protein interactions mediating the modulatory effect of cytoplasmic factors on these channels.

A search for evolutionary kinship among channels. Does the smaller hydrophobic domain of the inwardly rectifying potassium channel reflect the simpler design of an ancestral channel? To address this question we have aligned the M1, M2 and H5 domains of an inwardly rectifying potassium channel (ROMK1⁴) with sequences of other channels, including the γ -subunit of the mammalian amiloride-sensitive sodium channel³⁹ (γ -rENaC), a mechanosensitive channel from *E. coli*⁴⁰ (mScL), the bacterial high-affinity potassium transport protein KdpA¹⁹, a low affinity potassium transporter from *E. coli* (TrkG)⁴¹, the *Drosophila* voltage-gated potassium channel Shaker¹, the CFTR chloride channel³⁶ and the voltage-gated CIC-0 chloride channel from *Torpedo*³⁵ (Fig. 2).

rENaC contains three structurally related subunits, each with two widely separated hydrophobic segments, which show sequence similarity with the products of the *C. elegans* genes

	M1																					
γ -rENaC	L	L	W	I	A	F	T	L	T	A	V	A	L	I	I	W	Q	C	A	L	L	75
mscL	L	A	V	G	V	I	I	G	A	F	G	K	I	V	S	S	L	V	A	D	I	40
KdpA1	L	L	I	A	L	F	F	I	Q	Q	G	A	L	Q	N	F	L	P	Y	Q	A	204
KdpA2	L	T	A	L	A	I	L	V	T	P	T	L	V	L	M	G	A	A	L	M	M	437
TrkG1	I	I	I	V	M	F	W	I	L	F	S	V	I	S	A	F	L	W	I	D	S	95
TrkG2	S	V	W	S	F	F	F	L	Y	T	L	T	V	F	F	I	L	V	L	N	G	419
ROMK	V	F	I	T	A	F	L	G	S	W	F	L	F	G	L	L	W	Y	V	V	A	104
Shaker	L	G	L	L	I	F	F	L	I	G	V	V	L	F	S	S	A	V	Y	F	A	417
CFTR	L	Q	A	S	A	F	C	G	L	G	F	L	I	V	L	A	L	F	Q	A	G	240
CIC-0	A	F	V	P	V	F	N	L	G	A	V	L	G	R	F	V	G	E	L	M	A	438

	H5																					
γ -rENaC	S	P	A	N	S	I	E	M	L	S	N	F	G	G	Q	L	G	L	W	M	S	541
mscL	R	D	A	Q	G	D	I	P	A	V	V	M	H	Y	G	V	F	I	Q	N	V	83
KdpA1	A	S	Q	E	A	I	K	M	L	G	T	N	G	G	F	F	N	A	N	S	S	241
KdpA2	A	V	S	S	A	A	N	N	G	S	A	F	A	G	L	S	A	N	S	P	F	480
TrkG1	L	N	L	T	F	I	D	A	L	F	E	G	V	S	G	I	T	T	G	A	T	118
TrkG2	A	T	V	A	A	C	I	N	N	M	G	L	G	F	G	A	T	A	S	F	G	450
ROMK	A	F	L	F	S	L	E	T	Q	V	T	I	G	Y	G	F	R	F	V	T	E	152
Shaker	A	F	W	W	A	V	V	T	M	T	T	V	G	Y	G	D	M	T	P	V	F	453
CFTR	A	Y	V	R	Y	F	N	S	A	F	F	S	G	F	F	V	V	F	L	S	321	
CIC-0	T	H	A	V	S	T	A	V	I	C	F	E	L	T	G	Q	I	S	H	V	L	492

	M2																					
γ -rENaC	V	V	C	V	I	E	I	I	E	V	F	F	I	D	F	F	S	I	I	A	R	565
mscL	F	L	I	V	A	F	A	I	-	F	M	A	I	K	L	I	N	K	L	N	R	105
KdpA1	A	L	T	N	F	V	Q	M	-	L	A	I	F	L	I	P	T	A	L	C	A	269
KdpA2	A	F	C	M	F	V	G	R	-	F	G	V	I	I	P	V	M	A	I	A	G	506
TrkG1	R	A	Y	L	Y	R	S	Q	L	N	F	I	G	G	L	G	V	I	V	L	A	149
TrkG2	V	L	N	D	I	A	K	C	-	L	M	C	I	A	M	I	L	G	R	L	E	471
ROMK	I	F	L	L	I	F	Q	S	I	L	G	V	I	I	N	S	F	M	C	G	A	178
Shaker	V	G	S	L	C	V	V	A	G	V	L	T	I	A	L	P	V	P	V	I	V	479
CFTR	K	G	I	I	L	R	K	I	-	F	T	T	I	S	F	C	I	V	L	R	M	349
CIC-0	A	N	M	V	A	Q	G	L	Q	P	S	L	Y	D	S	I	I	Q	I	K	K	521

FIG. 2 Sequence alignment for the KdpA¹⁹ and TrkG⁴¹ potassium transport proteins in *E. coli*, the bacterial mechanosensitive channel mScL⁴⁰, the γ subunit of the mammalian epithelial amiloride-sensitive sodium channel³⁹ (γ -rENaC), the mammalian ROMK1 inwardly rectifying potassium channel⁴, the *Drosophila* Shaker voltage-gated potassium channel¹, the human CFTR chloride channel³⁶ and the *Torpedo* CIC-0 chloride channel³⁵. In the M2 alignment, boxed residues in Shaker^{16,27} and CFTR^{43,44} are known to affect ion permeation, and boxed residues of the epithelial sodium channel correspond to those implicated as pore-lining⁴². Number to the right of each sequence refers to the position of the last residue in the sequence. The *kch* gene product⁵⁰, which is highly conserved in *E. coli* and *Bacillus*, also fits in this alignment (its last residue in M1, 158; H5, 196; M2, 222). Because of its higher level of amino acid identity with ROMK1 (24%) and Shaker (32%) than the other channel sequences (average 10%), it is not included in the consideration for the probability. See Box 1 for probability calculation.

Probability calculations

To estimate the probability of ten random hydrophobic sequences of 22 residues each having the extent of amino acid identity shown in Fig. 2, we assume that the frequency of occurrence of each of the eight residues L,A,F,G,I,V,S,Y is 0.125. This should give an overestimate of the probability since all 20 amino acids are present in this alignment and the actual frequency for any one is less than 0.125.

$C_m^n = m!/n!(m-n)!$ = number of events that n out of m subjects are grouped together.

P_{10} = probability that all 10 residues at a specific position of the alignment are identical = $(0.125)^{10} \cdot 8 = 7.45 \times 10^{-9}$.

P_9 = probability that 9 of 10 residues at a specific position are identical = $10 \cdot (0.125)^9 \cdot (0.875) \cdot 8 = 5.22 \times 10^{-7}$.

P_8 = probability that 8 of 10 residues at a specific position are identical = $C_8^{10} (0.125)^8 \cdot (0.875)^2 \cdot 8 = 1.64 \times 10^{-5}$.

P_7 = probability that 7 of 10 residues at a specific position are identical = $C_7^{10} (0.125)^7 \cdot (0.875)^3 \cdot 8 = 3.07 \times 10^{-4}$.

P_6 = probability that 6 of 10 residues at a specific position are identical = $C_6^{10} (0.125)^6 \cdot (0.875)^4 \cdot 8 = 3.76 \times 10^{-3}$.

$P_{5,3}$ = probability that 5 are identical and 3 others are identical at a specific position = $C_5^{10} C_3^5 (0.125)^5 (0.125)^3 (0.75)^2 \cdot 8 \cdot 7 = 4.73 \times 10^{-3}$.

P_5 = Probability that 5 are identical at a specific position = $C_5^{10} (0.125)^5 (0.875)^5 \cdot 8 = 3.16 \times 10^{-2}$.

$P_5 - P_{5,3} = 2.68 \times 10^{-2}$ > probability that 5 are identical but less than 3 of the other 5 residues are identical.

$P_{4,3}$ = probability that 4 are identical and 3 others are identical at a specific position = $C_4^{10} C_3^6 (0.125)^4 (0.75)^3 \cdot 8 \cdot 7 = 4.73 \times 10^{-2}$.

$P_4 = C_4^{10} (0.125)^4 (0.875)^6 \cdot 8 = 0.184$.

$P_4 - P_{4,3} = 0.137$ > probability that 4 are identical and less than 3 of the other 6 are identical.

$P_{3,3} = C_3^{10} C_3^7 (0.125)^3 (0.125)^3 (0.75)^2 \cdot 8 \cdot 7/2 = 0.142$.

$P_3 = C_3^{10} (0.125)^3 (0.875)^7 \cdot 8 = 0.736$.

$P_3 - P_{3,3} = 0.594$ > probability that 3 are identical and less than 3 of the other 7 are identical.

M1 1 position, 8 identical (F)

1 position, 6 identical (L)

1 position, 4 identical (L) and 3 identical (G)

3 positions, 4 identical (A,L,V)

9 positions, 3 identical (I,F,A,F,F,F,L,A,A)

$$P_{M1} < C_1^{22} P_8 C_1^{21} P_6 C_1^{20} P_{4,3} C_3^{19} (P_4 - P_{4,3}) C_3^{18} (P_3 - P_{3,3})^9 = 7.07 \times 10^{-3}$$

H5 1 position, 10 identical (G)

1 position, 6 identical (A)

2 positions, 4 identical (G,A) and 3 identical (F,S)

1 position, 3 identical (S) and 3 identical (F)

7 positions, 3 identical (A,I,L,T,Y,F,F)

$$P_{H5} < C_1^{22} P_{10} C_1^{21} P_6 C_2^{20} (P_{4,3})^2 C_1^{18} P_{3,3} C_3^{17} (P_3 - P_{3,3})^7 = 7.14 \times 10^{-6}$$

(The estimated upper bound for the probability for the M2 alignment would be similar to that for M1, if no penalty is introduced for the gap in the M2 alignment.) This calculation significantly overestimates the upper bound for the probability and gives numbers much greater than 1 (10^{-3} for M1; 10^{-5} – 10^{-4} for H5) as the upper bound for the probability for various alignments of scrambled sequences.

mec-4, *mec-10* and *deg-1*, all thought to encode mechanosensitive channels^{39,42}. Figure 2 shows that the sequences of this family can be aligned with those of mScL and of the potassium channels. Moreover, in each of these sequences, an H5-like segment immediately precedes the final hydrophobic domain. KdpA and TrkG each contain two sets of M1, H5 and M2 segments (Fig. 2). The alignment also includes the last three hydrophobic segments in CIC-0 and the first ABC domain of CFTR.

For each of the ten aligned sequences, the pairwise amino-acid identity with each of the other nine sequences averaged about 12%, ranging from 9% to 14%. This may seem modest for such hydrophobic sequences, but in several positions the same amino acid is found in three or more sequences, and the calculation set out in Box 1 suggests that such a level of identity is unlikely to arise by chance ($P < 10^{-2}$ for M1; $P < 10^{-5}$ for H5).

Mutagenesis has implicated sequences in the H5 and/or M2 domain in ion transport by KdpA¹⁹, CFTR^{43,44}, the probable mechanosensitive channels of *C. elegans*⁴², and voltage-gated potassium channels^{11,13,16,17}. Cross linking studies indicate that the M2 domain lines the pore of voltage-gated calcium channels⁴⁵ (which belong to the same superfamily as voltage-gated potassium channels) and of the multidrug resistance P-glycoprotein⁴⁶ (which belongs to the ABC superfamily). Thus the hydrophobic segments in Fig. 2 appear to represent the basic design for a channel pore.

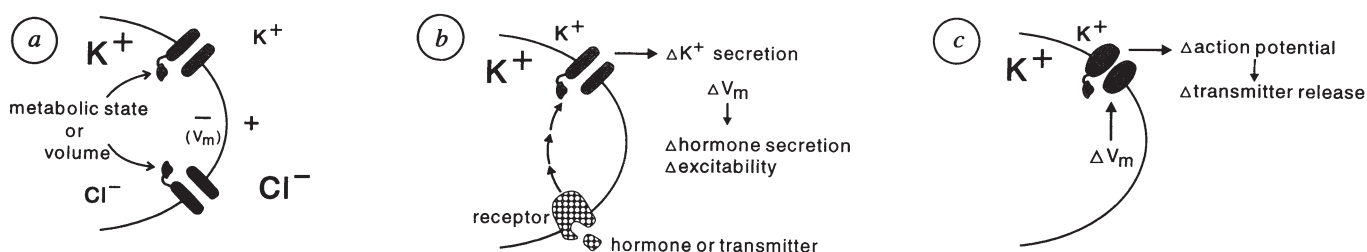


FIG. 3 Hypothesis on channel evolution. *a*, Channels with cytoplasmic gates sensitive to volume or metabolic state (e.g. ATP to ADP ratio) are postulated to appear early during evolution. *b*, The actively maintained asymmetric potassium distribution allows potassium channels to control membrane potential (V_m), thereby regulating hormone release (e.g. insulin release from the pancreatic β cells) and excitability (e.g. the rate of heartbeat). The activities of potassium channels in turn may be regulated by extracellular and intracellular messengers. *c*, For potassium channels to be regulated by membrane potential, membrane-embedded voltage sensors are added to detect the electrical potential across the membrane, allowing voltage-gated potassium channels to control the waveform and frequency of action potentials, and hence the amount of transmitter released.

Channel gating evolution

The ubiquity of a cytoplasmic gate in different ion channels, as well as the sequence similarity between these channels (Fig. 2), indicate that channels with a cytoplasmic gate may represent a primitive form of channel, from which other types, like the voltage-gated channels, have arisen. This idea is explored further here in the hope of stimulating further discussion.

One of the most basic demands on the primitive cell, the ability to tolerate alterations in surrounding tonicity without bursting, may involve the regulation of ion flow by cell volume (Fig. 3*a*). An ion channel may be able to detect stretch or tension in the membrane, as in the case of *mscL*⁴⁰. Mechanosensitivity may also involve interaction with cytoskeletal elements, as in the hair cells of the mammalian ear⁴⁷. Volume regulation the *CIC-2* chloride channel involves a cytoplasmic gate³⁵.

As a cell assumes additional functions, such as salt secretion or intercellular communication, it may be selectively advantageous to acquire ion channels that can regulate their activities according to the metabolic state of the cell (as measured by the ATP to ADP ratio, for instance), in a manner analogous to the regulation of chloride secretion by the CFTR or the control of insulin release by ATP-sensitive potassium channels. In addition to evaluating the intracellular metabolic state, such a channel could also acquire sensitivity to other intracellular or extracellular signals like calcium ions, cyclic nucleotides and G proteins (Fig. 3*a, b*).

Cytoplasmic gates are well suited for this purpose. Covalent linkage of the cytoplasmic gate to the channel is apparently unnecessary for gating, but it may facilitate coordinated expres-

sion and targeting, explaining the ball-and-chain design of gates like the inactivation gate of voltage-gated potassium channels.

Inwardly rectifying potassium channels, such as ATP-sensitive potassium channels and G protein-activated muscarinic potassium channels, may thus retain the pore and cytoplasmic gate of the ancestral channel. The activity of such channels is controlled by the electrochemical driving force for potassium, as well as intracellular signals and the metabolic state of the cell, but they are not gated by membrane potential *per se* (Fig. 3*b*).

To detect and respond to changes in the membrane potential, ancestral channels may have acquired membrane spanning segments (such as S4) that allow charges to be lodged in the hydrophobic interior of the membrane (Fig. 3*c*). To shield these charges from the lipids, additional membrane spanning segments such as S1–S3 may be necessary. Every third or fourth residue in the S1–S3 segments of voltage-gated potassium channels is hydrophobic but not conserved among family members, as in the lipid-facing transmembrane helices of the photosynthetic reaction centre, indicating that they may form the outer rim of the potassium channel (Fig. 1). The new gate, a voltage sensor, monitors the membrane potential and mediates the conformational changes leading to channel activation. The more ancient cytoplasmic gate may nevertheless have been retained and used to control the duration of channel activity. □

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Testing the iron hypothesis in ecosystems of the equatorial Pacific Ocean

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The idea that iron might limit phytoplankton growth in large regions of the ocean has been tested by enriching an area of 64 km² in the open equatorial Pacific Ocean with iron. This resulted in a doubling of plant biomass, a threefold increase in chlorophyll and a fourfold increase in plant production. Similar increases were found in a chlorophyll-rich plume downstream of the Galapagos Islands, which was naturally enriched in iron. These findings indicate that iron limitation can control rates of phytoplankton productivity and biomass in the ocean.

OVER 20% of the world's open ocean surface waters are replete with light and the major plant nutrients (nitrate, phosphate and silicate), yet standing stocks of phytoplankton remain low. The factors that limit phytoplankton growth and biomass in these high-nitrate, low-chlorophyll (HNLC) areas have been vigorously debated¹, but not resolved. The suggestion that increased phytoplankton production in HNLC areas could remove significant amounts of carbon dioxide from the atmosphere has renewed interest in this topic².

In some HNLC areas, it has been suggested that zooplankton grazing may contribute to the maintenance of low chlorophyll levels³. Strong turbulence at high latitudes may also mix phytoplankton below the critical depth, resulting in light-limitation of growth^{4,5}. We believe that in addition to these factors, micronutrient elements, such as iron, which are important catalytic components in a wide variety of electron transport and enzymatic systems, have the potential to limit phytoplankton production in HNLC areas⁶. There are several lines of evidence in support of this notion.

The development of trace-metal clean sampling techniques over the last two decades has revolutionized our understanding of trace-metal biogeochemistry in the oceans⁷. Open-ocean iron concentrations in surface waters exist at picomolar (10⁻¹² M) levels. These concentrations may be insufficient to support high phytoplankton biomass or maximum growth rates⁸. Addition of Fe to uncontaminated seawater samples has been shown to stimulate the growth of phytoplankton, especially diatoms⁹⁻¹⁶. Laboratory experiments have demonstrated that low iron levels may limit phytoplankton growth rates¹⁷⁻¹⁹. Moreover, techniques such as fast repetition rate (FRR) fluorometry have shown that photochemical energy-conversion efficiency is less than maximal in the equatorial Pacific and can be increased significantly in phytoplankton supplied with nanomolar (10⁻⁹ M) levels of iron^{20,21}. These experiments suggest iron availability may regulate ocean production in HNLC areas.

The extrapolation of results from shipboard and laboratory incubation experiments to whole ecosystems has, however, been strongly criticized²²⁻²⁴. Although small-scale experiments may accurately reflect *in situ* processes occurring at the chemical or first trophic level, bottle experiments, by design, do not accurately represent the nature or scale of the community response. Studies of nutrient limitation in lakes have demonstrated that

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incubations performed on small phytoplankton samples cannot easily be extrapolated to whole ecosystems. Mesocosm experiments with complete communities however, correlated highly with whole-lake responses to nutrient enrichments²⁵.

We believe that the effects of iron on phytoplankton growth in HNLC areas can be more confidently resolved by mesoscale

enrichments of whole ecosystems²⁶. Until recently however, enrichment experiments in the open ocean have not been feasible due to the difficulties in tracking a patch over time. The recent development of technology to measure ultra-trace concentrations of the inert gas sulphur hexafluoride, SF₆, has made it possible to mark a patch of sea water and track it for long

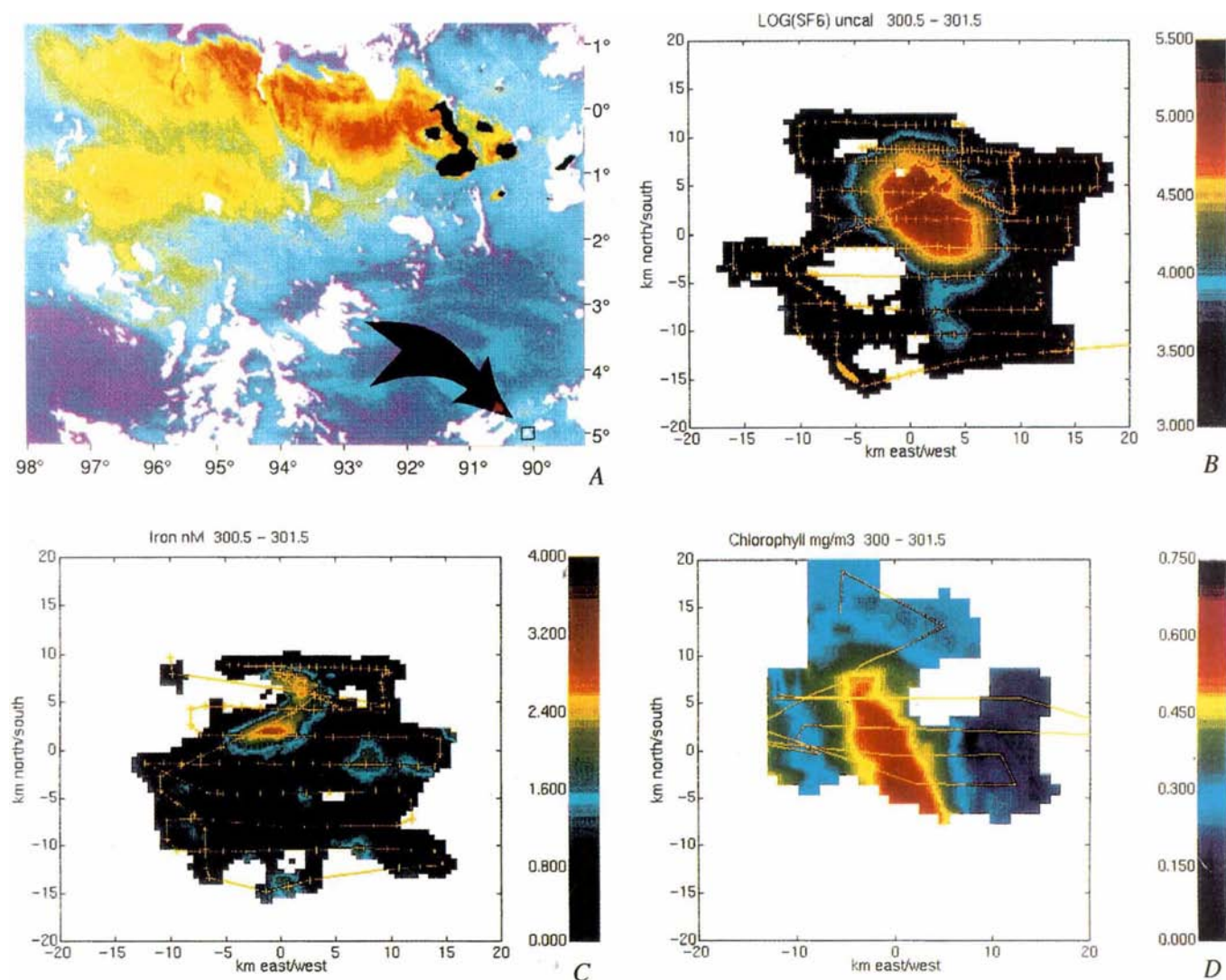


FIG. 1 A, False-colour image of the study site from the Coastal Zone Colour Scanner on the Nimbus 7 satellite, October 1983. This image indicates the chlorophyll-rich plume extending west of the Galapagos Islands (red colour indicates higher chlorophyll concentrations; image courtesy of G. Feldman). The arrow indicates the location and relative size of the study site at 5° S. B–D, Contour plots of the concentrations of SF₆, (B) YD 300.5–301.5, iron (C) YD 300.5–301.5, and chlorophyll (D) YD 300–301. All parameters in B–D are plotted relative to the GPS buoy which served as the centre of a lagrangian frame of reference used during the first part of the experiment. A total volume of 15,600 l of the iron solution (8,100 mol or 450 kg Fe) was dispensed. The SF₆ solution was prepared by dissolving 0.35 mol of SF₆ in sea water in a 2,300-l steel tank before departure from Miami. The SF₆ was pumped (1.4 l min⁻¹) together with the iron solution (12 l min⁻¹) into the propeller wash as the ship steamed at ~9 km h⁻¹. The ship's track was updated every 5 min relative to the central GPS buoy (see below) to correct for horizontal advection during the 24-h deployment. This strategy was estimated to produce an increase in the iron concentration within the 35-m mixed layer of 3.8 nM and an SF₆ concentration of 180 fM (ref. 51). Horizontal eddy diffusion was predicted to horizontally homogenize the streaks within 1 d of mixing, whereas overturn of the mixed layer would vertically homogenize the iron and SF₆ concentra-

tions every evening³⁶. The GPS buoy was interrogated every 5 min to determine the location of the ship relative to this central point. In addition, four WOCE drifter buoys equipped with ARGOS position transmitters were deployed at each corner of the patch. Updates of the ARGOS buoy positions could be received four times a day. All of the buoys were attached to holey sock drogues set at 10 m to reduce wind slippage relative to the enriched patch. A flow-through pumping system with an intake set at 3 m on the ship's bow was used to sample surface sea water. SF₆ measurements using electron-capture gas chromatography were used to determine the position of the enriched iron patch, relative to the GPS buoy. This information was used daily to correct the central lagrangian reference point based on the GPS buoy (that is, correction for buoy slippage). Surface sea water was supplied for iron analyses via an all-Teflon pumping system also with the intake on the ships' bow. Samples were acidified in-line (pH 3) before analyses resulting in the detection of dissolved iron, freshly precipitated colloidal iron, and much of the aged, colloidal iron (total dissolvable iron, DFe). DFe was analysed continuously using both colorimetric and chemiluminescent techniques (detection limit, 0.3 nM). Chlorophyll depicted in D represents that filtered and extracted from discrete samples taken along the ship's track. Chlorophyll concentrations were quantified using standard fluorescence techniques on board ship.

periods of time^{27,28}. Thus, when combined with iron enrichment, the SF₆ tracer provides the potential to assess ecosystem level responses to added iron²⁹.

The region south of the Galapagos Islands was proposed as the most favourable place for such an iron-enrichment experiment³⁰. In this paper, we report the initial results of a mesoscale iron-enrichment experiment performed in this area of the equatorial Pacific. Additionally, the waters both upstream and downstream of the Galapagos Islands were studied to further evaluate the potential role of iron in regulating produc-

tion. We found substantial and convincing evidence to indicate that iron additions to these HNLC regions increases phytoplankton productivity and biomass.

Open ocean iron fertilization experiment

In mid-October 1993, the RV *Columbus Iselin* occupied an area approximately 500 km south of the Galapagos Islands to begin the enrichment experiment (Fig. 1A). Ambient iron concentrations in this area were of the order of 0.06 nM (ref. 31). An area of about 64 km² was enriched with iron to a concentration of

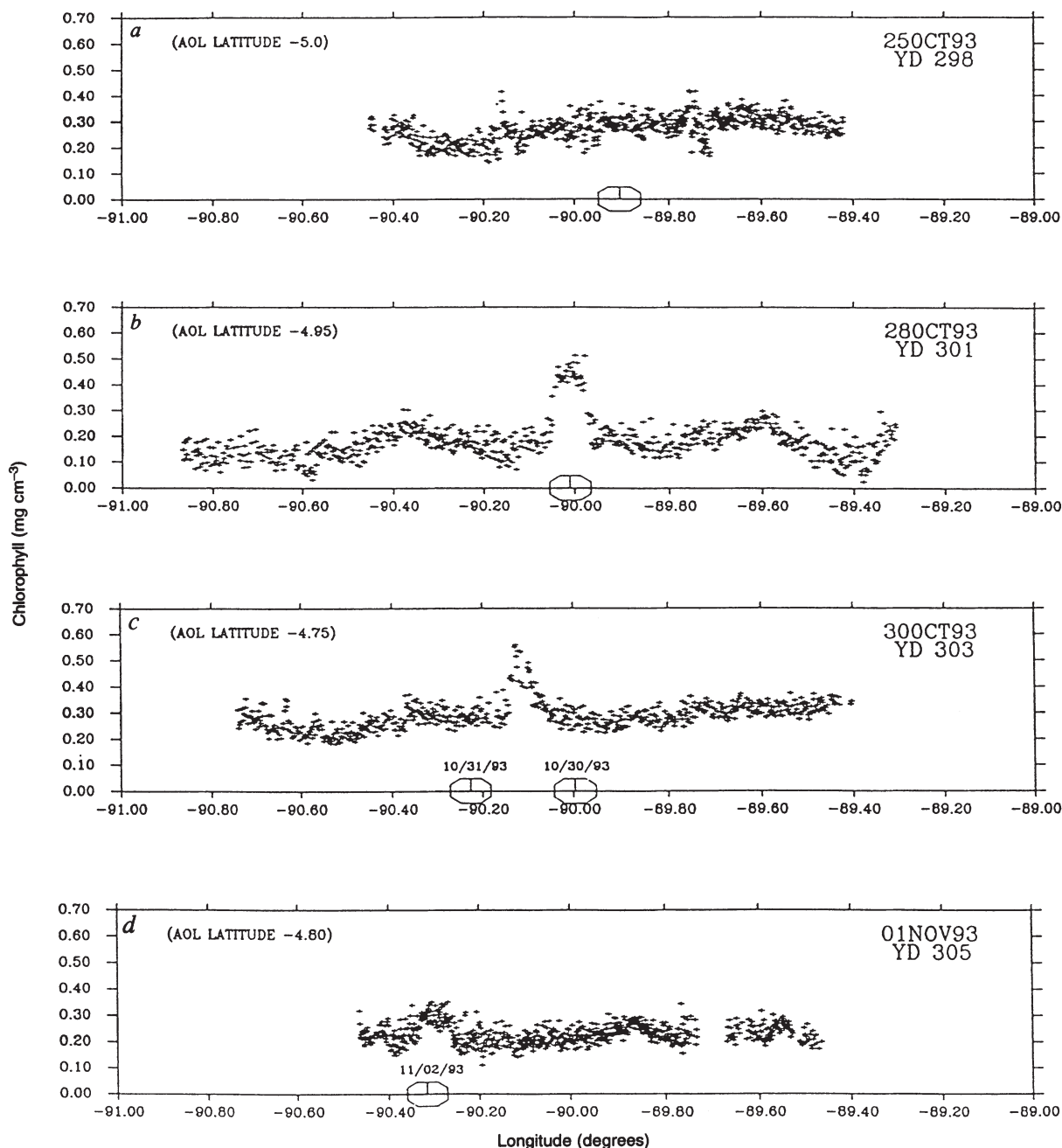


FIG. 2 a–d, The NASA airborne oceanographic LIDAR flown in a P-3 Orion aircraft (from Goddard Space Flight Center/Wallops Flight Facility) provided pigment measurements determined by laser-induced chlorophyll fluorescence on YD 298 (a), YD 301 (b), YD 303 (c) and YD 305 (d). For each mission day a single representative east–west flightline was selected from numerous lines flown over the patch. The latitude of the horizontal flightline is given in each panel. The longitude of the most contemporaneous central buoy position is indicated by the circle and

date at the bottom of each panel. The flight legs were ~180 km long and were flown in a star pattern centred on the GPS buoy⁵². The surface manifestation of the patch was seen at (or very near) the buoy positions on each day after the initial deployment on YD 298. Fluorescence signals showed a rapid initial response followed by a decay in signal strength as the experiment progressed. This result is consistent with the measurements of iron and photochemical energy conversion efficiency⁴².

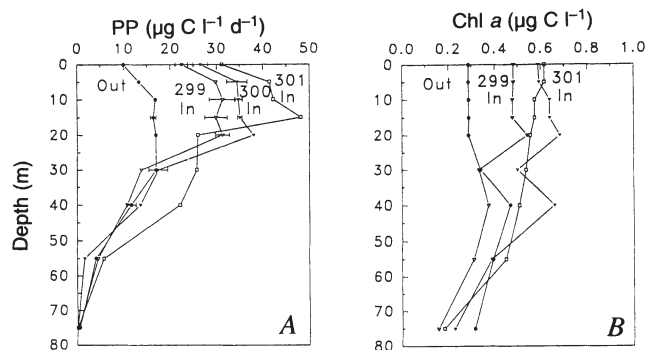


FIG. 3 Vertical profiles, for the 3 days following fertilization, of primary production, PP, (A) chlorophyll *a* concentrations, Chl *a*, (B) as a function of time inside and outside the patch. Outside values are depicted for YD 299. Primary production was measured using H^{14}CO_3 uptake determined at various light levels, in incubations on board the ship. Chlorophyll was determined from filtered and extracted samples as in Fig. 1D. The errors associated with the chlorophyll analyses are generally $<0.02 \mu\text{g C l}^{-1}$. The depth to which the water column was enriched was ~ 35 m up to YD 301 (just before subduction). It is in the upper 35 m that the differences are most pronounced. Productivity and chlorophyll both converge by 75 m.

~ 4 nM. Iron at this level, in bottle experiments, is sufficient to cause large increases in chlorophyll and complete depletion of the available major nutrients within 5 to 7 days. The enriched patch was tracked for 10 days and monitored for changes in biological, chemical and physical parameters.

To ensure that changes in properties within the patch could be attributed to the presence of iron and not to natural variation of the area in general, the study area was surveyed on year days (YD) 295 to YD 297 to determine its biological, chemical and physical heterogeneity. The initial survey, as well as later stations outside the patch, showed mixed-layer plant nutrient concentrations of nitrate, phosphate, silicate and ammonium to be $10.8 \pm 0.4 \mu\text{M}$, $0.92 \pm 0.02 \mu\text{M}$, $3.9 \pm 0.1 \mu\text{M}$ and $0.21 \pm 0.02 \mu\text{M}$ respectively, with a chlorophyll concentration of $0.24 \pm 0.02 \mu\text{g l}^{-1}$. These values are typical of the equatorial HNLC region^{32,33}. The low-chlorophyll conditions were substantiated throughout the region by overflights of the NASA airborne oceanographic lidar (AOL) optical laboratory before the experiment (Fig. 2). Iron (450 kg) was added to the patch as a 0.5 M Fe(II) solution in sea water at approximately pH 2, together with 0.35 mol of the inert chemical tracer, SF_6 .

Four tracking strategies were employed to maintain contact with the iron enriched area. (1) The iron deployment and initial sampling were performed about a central lagrangian reference point located by a drogued buoy equipped with a Global Positioning System (GPS) receiver interfaced to a VHF packet radio transmitter and receiver. (2) SF_6 was released in constant ratio to the iron injected into the patch. This inert tracer was monitored continuously with detection limits of less than 10^{-16} M. (3) The NASA AOL conducted overflights on YD 298, YD 301, YD 303 and YD 305 to assess the large-scale effects of iron on surface-water chlorophyll in the patch as compared to the surrounding region (Fig. 2). (4) The concentration of iron in the patch was determined continuously throughout the experiment^{34,35}.

The patch was sampled from YD 298.9 to YD 302.0 using surface pumping systems as the ship steamed through the study area. Each day, one major hydrographic station was occupied

within the patch and one outside the patch at a time near local noon. Samples were collected at these stations for analysis of primary productivity, species composition, chlorophyll, FRR fluorescence, pigments (by high-pressure liquid chromatography), nutrients, particulate organic carbon (POC), particulate organic nitrogen (PON), dissolved organic carbon (DOC), dimethylsulphide (DMS), dimethylsulphoniopropionate (DMSP), dissolved and particulate trace metals and halocarbons as well as hydrographic and CO_2 system parameters. The concentration of the SF_6 tracer, together with the ship's position relative to the GPS buoy, were used to distinguish stations inside the patch from those outside.

Physical behaviour of the patch. The physical coherence of the patch was one of the greatest concerns in performing the experiment. One potential problem was that as the patch spread by turbulent diffusion, it would disperse into long streaks as it crossed eddy or frontal boundaries. It was, however, remarkably stable. As predicted, the added iron and SF_6 were distributed rapidly throughout the mixed layer by horizontal eddy diffusion and convective overturn. The patch remained as an intact unit from YD 298 to at least YD 302, expanding to become a rectangle of approximately 8×12 km.

Late on YD 302 the core of the patch was subducted beneath a low-salinity front to a depth of 30–35 m, where it was confined in a 5–10 m layer at the top of the thermocline. The patch was still detectable after its subduction by the SF_6 signal, the distinct salinity and low light transmission³⁶. SF_6 concentrations did not decrease after subduction. The highest values of SF_6 found each day were a constant 40–50 fM (femtomolar, 10^{-15} M) over the entire 9-day duration of the experiment. The constancy of the SF_6 concentration suggests that unfertilized waters did not penetrate into the core of the patch even after subduction.

Iron chemistry. Dissolvable iron concentrations (DFe) as high as 6.2 nM were measured in the core of the patch within 4 hours of fertilization. DFe decreased rapidly on the first day due to night-time convective mixing of the water column, with the highest values on the subsequent day being 3.6 nM, in good agree-

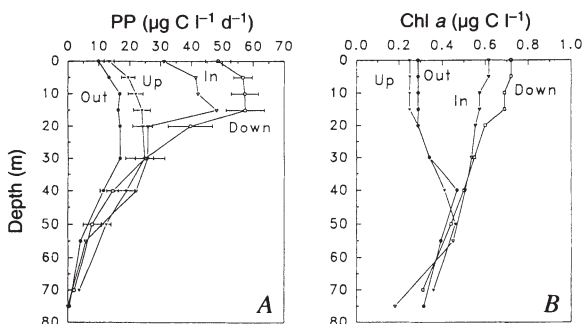


FIG. 4 Comparison of vertical profiles of primary production (A) and chlorophyll *a* concentrations (B) for stations inside and outside the fertilized patch, and stations upstream (westward) of the Galapagos Islands and downstream (eastward) of the Galapagos Islands.

ment with predicted concentrations (3.8 nM Fe). Concentrations of DFe in the core of the patch decreased $\sim 15\%$ each day. Despite this decrease, contour plots of iron and SF_6 in the surface layer were still in good spatial agreement on YD 301, 3 days after fertilization (Fig. 1B, C). Iron dropped below our detection limit (0.3 nM) in the subducted layer by YD 303.

Previous studies indicate that three distinct pools of iron were formed on injection; dissolved Fe(III) , colloidal Fe(III) and cellular Fe. Most of the Fe(II) injected into the patch was oxidized within minutes to Fe(III) ³⁷ and colloidal iron oxyhydroxides should have precipitated with a first-order rate constant of 0.1 h^{-1} (ref. 16). Biological uptake of the dissolved Fe(III) proceeded rapidly (see below). Of the three iron forms, colloidal Fe(III) , especially when aged, is not thought to be bioavailable. Laboratory experiments, however, indicate that these colloids would remain suspended in the surface waters (K.H.C. *et al.*, unpublished observations) where they could be photoreduced and rendered bioavailable^{16,38}. A computer model of iron photochemistry¹⁶ suggests that once the patch was subducted, there would be insufficient light to maintain the bioavailable pool of dissolved Fe(III) at concentrations above our shipboard detection limits.

Chemical response. Nutrient measurements indicated little or no systematic difference in nitrate, phosphate and silicate concentrations within the mixed layer between inside and outside stations. The ratio of nitrate uptake to chlorophyll production in Fe incubation experiments is approximately 1 mol NO_3 per g chlorophyll (ref. 6). Thus a chlorophyll increase of $0.5 \mu\text{g l}^{-1}$ should have been accompanied by a $0.5 \mu\text{M}$ decrease in nitrate. Because initial nitrate concentrations were $10.8 \mu\text{M}$ and the day-to-day variance in the nitrate measurements was $0.4 \mu\text{M}$ (1σ) a $0.5 \mu\text{M}$ decrease would have been at our limit of detection. Phosphate and silicate also showed no definitive change over this time period inside the patch.

Ammonium, however, showed a consistent difference between inside and outside stations. After fertilization, the mean ammonium concentrations measured inside the patch from YD 299 to YD 302 were $0.10 \pm 0.07 \mu\text{M}$ (95% confidence interval) lower than the values observed outside the patch. An ammonium maximum with values near $0.45 \mu\text{M}$ was regularly found near the base of the mixed layer outside the patch. This maximum decreased within the fertilized patch and, with one exception, no ammonium concentrations higher than $0.12 \mu\text{M}$ were found inside the patch. Preferential uptake of ammonium³⁹ (especially by the picoplankton, the primary ammonium utilizers) would be expected as physiological rates increased.

The measurements of CO_2 fugacity and total CO_2 in the patch were significantly lower than those observed outside the patch⁴⁰. These changes were apparent within 2 days of the iron release and are lower than expected, but consistent with the drawdown in major plant nutrients. Particulate DMSP, integrated throughout the water column, showed a significant increase (50–

80%) in the fertilized patch. DMSP is produced by phytoplankton and decays to produce DMS ⁴¹. There were no clear changes in DMS concentration in surface waters ($2.5 \text{ nM} \pm 0.4$), which is not surprising considering the duration of the study compared to the time needed to degrade DMSP to DMS. Preliminary analysis of low-molecular-weight halocarbon data suggests that there was some increase in the concentration of methyl iodide inside the patch relative to outside stations.

Biological response. Biological parameters measured outside the patch during the course of the experiment remained similar to those measured within the patch region before the addition of Fe. Photosynthetic energy conversion efficiency (relative fluorescence) was the first biological response detected⁴² after Fe addition. Relative fluorescence dramatically increased by the first sampling transect through the patch, indicating a large physiological response within the first 24 hours (the period required to fertilize the patch)⁴².

Maximum primary production values in the mixed layer were initially $10\text{--}15 \mu\text{g C l}^{-1} \text{ d}^{-1}$ (R.B., personal communication). After fertilization, productivity increased monotonically to values of $48 \mu\text{g C l}^{-1} \text{ d}^{-1}$ on YD 301 (Fig. 3A). This 3–4 times increase in productivity was observed in all size fractions indicating an overall enhancement of rates in both small and large phytoplankton. Chlorophyll increased nearly three-fold by YD 301 from $0.24 \mu\text{g l}^{-1}$ to maxima consistently over $0.65 \mu\text{g l}^{-1}$ (Figs 2, 3B). NASA overflights confirmed the increase in chlorophyll using Lidar laser fluorescence measurements of the surface layer (Fig. 2).

Preliminary microscopic examination of water collected within the patch indicated an increase in biomass in all classes counted (cyanobacteria and protists). Total autotrophic biomass (excluding Prochlorophytes) calculated from cell numbers and volumes^{43–45}, increased from 16 to $33 \mu\text{g C l}^{-1}$. The largest contributors to the plankton biomass were *Synechococcus*, red fluorescing picoplankton and autotrophic dinoflagellates. Diatoms were a small fraction of the total plankton biomass (17%) yet showed increases similar to other groups. Heterotrophic biomass increased from 4.7 to $7.3 \mu\text{g C l}^{-1}$ and was dominated by heterotrophic dinoflagellates and ciliates. Prochlorophytes were not counted microscopically but preliminary flow cytometric analyses indicate little difference in cell numbers between stations inside and outside the patch.

Galapagos plume study

Satellite imagery collected with the Coastal Zone Colour Scanner on the Nimbus 7 satellite shows a plume of high-chlorophyll water that is found regularly to the west of the Galapagos Islands in association with the nutrient-rich, westerly flowing, south equatorial current⁴⁶ (Fig. 1A). Hydrographic surveys also have shown anomalously high chlorophyll concentrations and depleted nitrate concentrations over the Galapagos platform and downstream (westward) of the islands⁴⁷. It has been suggested

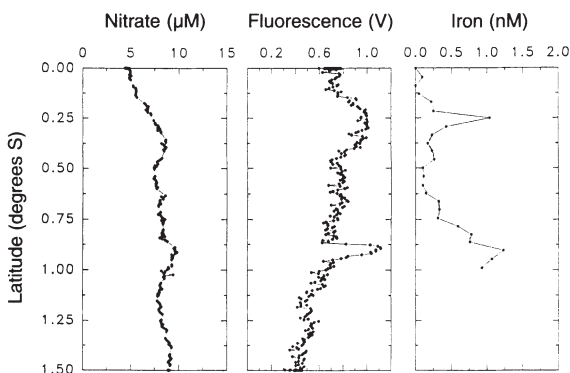


FIG. 5 Nitrate concentration, fluorescence intensity and iron concentrations as a function of latitude on a surface-water transect, along $90^\circ 45' \text{ W}$, from $1^\circ 30' \text{ S}$ to the Equator.

that these elevated chlorophyll levels are the result of iron derived from the island platform⁴⁷, but metal distributions in the Galapagos region are unknown. A series of stations were, therefore, occupied both upstream (to the east) and downstream (to the west) of the Galapagos Islands. Detailed trace-metal profiles were determined, as well as onboard analyses of the same parameters measured in the patch study. Surface-water iron, nitrate, CO₂ fugacity and fluorescence were also measured on transits between stations. The purpose of this sampling strategy was to determine if detectable anomalies of iron were present and, if so, their relationship to increased levels of chlorophyll and primary production in the downstream plume.

Survey results. The Galapagos Islands lie near a sharp equatorial front that separates low-nutrient, oligotrophic waters to the north from high-nutrient waters to the south. There are strong horizontal gradients as a result. Waters immediately around the Islands, during our survey, had nitrate concentrations greater than 10 μM at the surface. Chlorophyll concentrations and rates of primary production were significantly elevated at stations downstream, when compared to upstream (eastward, Fig. 4).

Surface-water iron concentrations were about 0.06 nM upstream of the Islands. Iron concentrations as high as 1.3 nM were detected on a transect across the downstream plume, from 1° 30' S to 0° along 91° 45' W (Fig. 5). These high concentrations appeared to be associated with upwelled water that was in contact with the shelf and has higher salinity and lower temperature than the surrounding surface water masses. Elevated chlorophyll fluorescence signals were also associated with the high iron concentrations (Fig. 5). The highest iron concentrations (3 nM) were found between the islands of Isabella and Fernandina. These concentrations were associated with chlorophyll levels in excess of 13 $\mu\text{g l}^{-1}$, near-maximum relative fluorescence and nearly complete depletion of nitrate.

Extracted surface chlorophyll concentrations in the downstream plume were typically near 0.7 $\mu\text{g l}^{-1}$, which is about 3 times higher than in the upstream water mass (Fig. 4B). However, large increases in chlorophyll fluorescence signals, which are indicative of even higher chlorophyll concentrations, were recorded by the shipboard fluorometer and the aircraft Lidar system on the downstream meridional transects through the plume^{38,48}. These high fluorescence signals were regularly associated with frontal features characterized by lower temperatures, higher salinities and higher nitrate concentrations. The highest fluorescence signals always occurred at the interface between these upwelled water masses and the surrounding water. Particulate DMSP and DMSO levels were also significantly higher in the plume than at stations upstream, whereas differences in DMS levels were small.

Discussion

Our results demonstrate a direct and unequivocal biological response of the equatorial Pacific ecosystem to added iron. The response observed in the fertilization experiment was similar in magnitude and character to the increased production and chlorophyll found in the Galapagos plume. Until this study, there were no measured differences in chemical constituents which could explain higher biomass west of the islands. The presence of elevated concentrations of iron in the downstream plume is consistent with the hypothesis that the high chlorophyll concentrations are supported by iron, originating from the Galapagos platform.

Concentrations of chlorophyll as well as rates of primary productivity were up to a factor of 3–4 higher in both the patch and the downstream plume compared to outside the patch and upstream of the islands. The rapid increase in photosynthetic energy conversion efficiency in all size classes of phytoplankton in the patch confirm that at least some members of the natural phytoplankton populations are physiologically limited by lack of available iron⁴². Chlorophyll-specific rates of primary production showed a smaller change. These rates of primary production

are less sensitive to iron availability because the carbon/chlorophyll ratio changes on the addition of iron. Moreover, laboratory studies show a three-fold increase in the carbon/chlorophyll ratio as cells become Fe-limited²⁰. Carbon/chlorophyll ratios (wt/wt) in the patch decreased from an initial value of 54 to 38 by YD 300. Productivity on a per unit carbon basis, as determined by estimates of cell biovolume, indicate an initial doubling time of 1.0 d⁻¹. By YD 301 the doubling time was 1.5 d⁻¹, a 50% increase. This indicates that the cells grow faster on a per unit carbon basis when iron is present.

Dimethylsulphoniopropionate in the particulate phase also increased within the patch relative to waters outside the patch. This compound serves an osmoregulatory function (or possibly as a cryoprotectant) in certain classes of phytoplankton. It degrades to DMS, a volatile sulphur-containing gas that contributes to non-seasalt sulphur in the atmosphere. The production of these compounds in the iron-enriched patch is consistent with the ice-core record, which links greater iron availability to increased phytoplankton production during glacial times⁴⁹.

There was evidence for increased grazing within the fertilized patch. Microscopic examination of samples collected inside and outside the patch indicate that microheterotrophic biomass increased by ~50% over the course of the patch experiment. This increase was rapid and appeared to level off quickly. The corresponding average increase in grazing pressure (assuming grazing rate ($\mu\text{g C d}^{-1}$) = $2.5 \times$ heterotrophic biomass; F.C. and K.B., personal communication), was approximately 4.5 $\mu\text{g C l}^{-1} \text{ d}^{-1}$.

The biomass increase (excluding Prochlorophytes), estimated from microscopic counts of cell numbers and volumes, was 16.4 $\mu\text{g C l}^{-1}$ (a doubling). If the pool of inorganic carbon decreased by an equivalent amount, then the fugacity of CO₂ in the mixed layer should have decreased by about 3 μatm in the fertilized patch. This finding is low but consistent with the measurements of Watson and co-workers, indicating that some carbon may have been exported or converted to DOC⁴⁰. When the increased effects of grazing are considered, the gross biomass increase must have been about 60 $\mu\text{g C l}^{-1}$. Most of this increase (43 $\mu\text{g C l}^{-1}$) was consumed over the 9-day experiment by grazers. These values are conservative in that they exclude consideration of mesozooplankton grazing. Qualitative observations of filters and plankton tows suggest that the mesozooplankton also increased, which presumably occurred when vertically migrating plankton encountered the high biomass in the patch and remained there to feed.

All biological indicators confirmed an increased rate of phytoplankton production in response to the addition of iron. The geochemical effect of iron enrichment was, however, small in both studies relative to the biogeochemical effect that could have been achieved if all the major nutrients were consumed. Nonetheless, the similarity in chlorophyll and productivity profiles measured between the fertilized patch and the downstream Galapagos plume suggests that similar mechanisms control the ecosystem response to added iron. There are several hypotheses that could explain why nutrients were not completely consumed and chlorophyll did not increase more dramatically. (1) Although iron did stimulate initial growth, the depletion of another micronutrient prevented further growth. (2) Grazing increased in the patch and plume environments as a result of increased production and the systems rapidly reached steady state. (3) Iron was lost from the system due to colloidal aggregation and/or sinking of larger particles containing iron.

First, we do not think that the limitation of the response to added iron results from the depletion of another trace nutrient. If that were the case, trace-metal clean shipboard incubations would not result in complete consumption of the major nutrients, but they do. Although there is no significant difference in dissolved Zn, Cu, Ni, Co or Cd between inside and outside stations, we cannot rule out the possibility that *in situ* growth resulted in the limitation of phytoplankton growth by other

bioactive trace metals due to biological uptake and re-partitioning of these resources. This possibility seems unlikely over these short timescales and the magnitude of the biological response.

Second, increased grazing pressure must have exerted some control on the rate of biomass increase. Yet grazing does not seem to be a likely mechanism for preventing phytoplankton from completely consuming the available major nutrients in the patch and downstream of the Galapagos Islands. Other investigators have also seen large depletions of major nutrients at stations over other regions of the Galapagos platform⁴⁷. Although reduced grazing in bottle experiments is likely, there is no reason to expect an equivalent reduction in grazing pressure, especially by microheterotrophs, over shelf regions where high chlorophyll concentrations were observed.

Third, the available data suggest that nutrient consumption and phytoplankton growth is limited in the patch and downstream of the Galapagos Islands because of a loss term that occurs in open waters but not in bottles or in shallow shelf regions. This loss term might be sinking of larger phytoplankton. If sinking keeps the diatom population low, then the absolute rates of nutrient uptake will be limited because phytoplankton growth is a first-order process. Iron would also be lost much more rapidly from open ocean systems than from bottles or shallow waters. In shallow waters, sinking iron is trapped in a nepheloid layer near the bottom. Concentrations in the nepheloid layer along continental margins can be greater than 10 nM (ref. 8). Upwelling of iron-enriched water from a shallow nepheloid layer will continually resupply the euphotic zone with iron. The biological similarity of shipboard iron enrichment experiments and stations over the Galapagos platform and Bolivar canal suggest that it is the elimination of the loss term for iron (that is, continuous supply of iron) that may be responsible for complete utilization of nutrients.

Vertical profiles of iron in the equatorial Pacific show a nutrient-like distribution with concentrations increasing at greater depth. However, the highest dissolved-iron values found in the equatorial Pacific (3°S, 140°W) are 0.55 nM at depths of 2,400 m (ref. 31). The depth of the source for upwelled water that reaches the surface along the equator is less than 150 m (ref. 50). Iron concentrations at this depth are less than 0.2 nM. Upwelling of water from the Equatorial undercurrent could not

produce the large iron concentrations that we observed in the meridional transect and at the Bolivar canal, unless it is dramatically enriched by contact with the island platform. This must account for the iron concentrations greater than 1 nM observed near the Islands, and ocean currents are, therefore, the most plausible mechanism for the transport of island derived materials to the plume.

The residence time of bioavailable iron added to surface waters must be very short. Iron disappeared rapidly from the patch and was also not detected far from the presumed source downstream of the islands. This indicates that, within a given parcel of water, both the patch and plume systems reflect a transient addition of iron rather than a sustained addition, characteristic of bottle enrichments and shallow shelf stations. With a transient addition, only a few cell divisions are possible before the iron is removed from the system. Continual supply must occur to sustain production. This probably accounts for the low plateau that was reached in the biological response to the iron-addition experiment and the relaxation in relative fluorescence within the fertilized patch⁴², but we cannot yet definitively rule out other factors.

We have shown that it is possible to enrich an area of the open ocean with iron and track it for many days. This capability can be used to conduct refined experiments that will address issues of grazing and iron loss, which will be coupled to continuous or semicontinuous enrichment experiments. Given the relatively cohesive nature of the patch that was produced, it seems possible to create and track even smaller patches and instrument these patches to obtain better estimates of carbon export. Open-ocean manipulative experiments are, therefore, possible and ecological studies in the open ocean are no longer limited to passive observations and bottle experiments. This, we feel, may change significantly the way geochemical and ecological studies are performed in the ocean.

Finally, we wish to make it clear that the purpose of these experiments is to understand the nature of the controls on productivity and ecosystem function in HNLC waters. Such experiments are not intended as preliminary steps to climate manipulation. We also emphasize the transient nature of these experiments which indicate that the impact of these manipulations is erased in a very short time, because of the short residence time of iron and the rapid food web response. □

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A genome-wide search for human type 1 diabetes susceptibility genes

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We have searched the human genome for genes that predispose to type 1 (insulin-dependent) diabetes mellitus using semi-automated fluorescence-based technology and linkage analysis. In addition to *IDDM1* (in the major histocompatibility complex on chromosome 6p21) and *IDDM2* (in the insulin gene region on chromosome 11p15), eighteen different chromosome regions showed some positive evidence of linkage to disease. Linkages to chromosomes 11q (*IDDM4*) and 6q (*IDDM5*) were confirmed by replication, and chromosome 18 may encode a fifth disease locus. There are probably no genes with large effects aside from *IDDM1*. Therefore polygenic inheritance is indicated, with a major locus at the major histocompatibility complex.

MANY common diseases such as diabetes, ischaemic heart disease, asthma and schizophrenia are likely to be aetiologically and genetically heterogeneous, where the same clinical disorder is caused by different susceptibility genes and environmental factors. The autoimmune disease type 1 diabetes has been shown to be a polygenic trait in mice¹⁻³, with the major locus encoded by the major histocompatibility complex (MHC) and at least nine other loci contributing to disease development. In humans, two chromosome regions show association with, and linkage to, type 1 diabetes: the MHC HLA region on chromosome 6p21 (*IDDM1*)^{4,5} and the insulin gene region (*INS*)⁶⁻¹¹ on chromosome 11p15 (*IDDM2*; the *CD3E* locus on chromosome 11q23 was previously designated *IDDM2* (ref. 12) but we have shown that this gene is not significantly associated with, or linked to disease (L.E.P. and J.A.T., manuscript in preparation)). The weight of evidence for *IDDM2* has come from case-control^{6,9,10} and intrafamilial association^{7,8,11} studies, but linkage has also been demonstrated⁹ and replicated¹⁰.

The degree of familial clustering of a disease (λ_s) can be estimated from the ratio of the risk for siblings of patients and the population prevalence¹³. For type 1 diabetes, λ_s is about 15 (6%/0.4%), but *IDDM1* and *IDDM2* alone do not account for the observed degree of familial clustering¹³. The λ_s for a particular disease locus can be estimated by calculating the ratio of the expected proportion of affected sibpairs sharing zero alleles identical-by-descent (IBD), which is 0.25, and the observed proportion¹³. In type 1 diabetic multiplex families (data from this study), λ_s for *IDDM1* is 3.1 ($z_0=0.08$; z_2 , z_1 and z_0 are the probabilities for sharing 2, 1 or 0 alleles IBD, respectively) and the λ_s for *IDDM2* is 1.3 ($z_0=0.19$). From these λ_s values it can be calculated¹³ that *IDDM1* and *IDDM2* contribute 42% and 10%, respectively, to the familial clustering of the disease.

A significant excess of alleles shared IBD in affected sibpairs is taken as evidence of genetic linkage. This approach does not require prior knowledge of disease gene frequencies or mode of inheritance¹⁴. The efficiency of the affected sibpair method can

be increased by using a maximum likelihood ratio test to obtain data from families that are not fully informative¹⁵, and by restricting the sharing probabilities to $z_0 \geq 0$, $z_1 \leq 0.5$ and $z_1 \geq 2z_0$ (ref. 16). Under these restrictions, a maximum lod score (MLS) ≥ 2.3 ($P \leq 0.001$) is taken to indicate significant evidence of linkage¹⁶.

So far, successful dissection of complex traits by genome-wide linkage mapping has been restricted to experimental organisms such as tomatoes¹⁷ and mice^{1,18}. The characterization of microsatellite marker loci by polymerase chain reaction (PCR)¹⁹, construction of high-resolution human genetic linkage maps²⁰, and the application of fluorescence-based, automated DNA fragment-sizing technology²¹⁻²³ have now greatly facilitated human genome-wide exclusion mapping, as demonstrated here.

Families and mapping strategy

Presence of insulinitis in human diabetic pancreata²⁴, the level and frequency of autoantibodies, and the strength of the HLA association are correlated with the age-of-onset of type 1 diabetes²⁵⁻²⁷. Furthermore, the persistence of plasma C-peptide, an indicator of residual pancreatic β -cell function, is more common in cases diagnosed over the age of 16 years²⁸. To reduce the level of aetiological heterogeneity and thus increase the power of our study, recruitment of families in the United Kingdom was restricted to nuclear, multiplex families in which at least one sibling was diagnosed under age 17 years and the other under age 29 years²⁸. Ethnic origin of the families was also an important criterion²⁹. The grandparents of all UK families were Caucasian and born in the UK³⁰.

A two-stage approach³¹ was employed to map genes for type 1 diabetes²⁹. First, a total genome screen by analysis of 289 microsatellite marker loci in 96 UK sibpair families (UK 96) was carried out. Then, two additional data sets comprising 102 UK sibpair families (UK 102) and 84 USA sibpair families (USA 84) were used for further analysis of marker loci with some positive evidence of linkage to type 1 diabetes (MLS ≥ 1 ,

$P < 0.05$). The USA sibpairs were selected according to our age-of-onset criterion from the Human Biological Data Interchange (HBDI) repository of type 1 diabetic multiplex families. These families were all Caucasian but of diverse ethnic origin³².

As *IDDM1* is a major locus, sibpairs were subdivided into two groups by IBD status at HLA: those sharing two HLA alleles or haplotypes IBD (that is, families showing stronger evidence of linkage to HLA) and those sharing either one or zero alleles. Sibpairs can also be subdivided according to their HLA type⁴. This strategy is computationally simple, and is, in principle, a stepwise version of simultaneous search³³, which was devised to facilitate detection of genes in genetically heterogeneous traits.

Stage 1: Analysis of 96 UK families

The microsatellite marker locus *TNFA*, located in the HLA class III region of the MHC and coincident with *IDDM1*, showed striking evidence of linkage to type 1 diabetes in the UK 96 families ($MLS = 7.3$; $\lambda_s = 2.5$; Fig. 1). MLS values approaching the magnitude of those obtained on chromosome 6p were not observed for any other chromosome (Fig. 1). Marker loci located up to 20 centimorgans (cM) away from the *TNFA* locus were

also seen to have MLS values exceeding 1.0 (Fig. 1; MLS value equal to 1.0 was chosen as an experimental threshold). Therefore, by taking a 20 cM radius on each side of every marker locus, we can exclude empirically at least 97% of the genome for genes with effects equivalent to the *HLA*-encoded *IDDM1* locus.

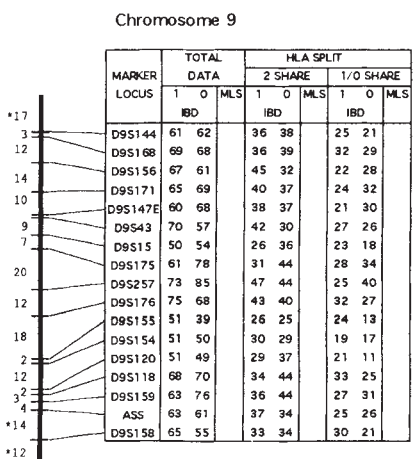
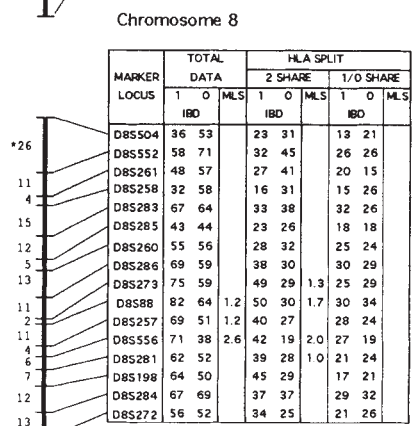
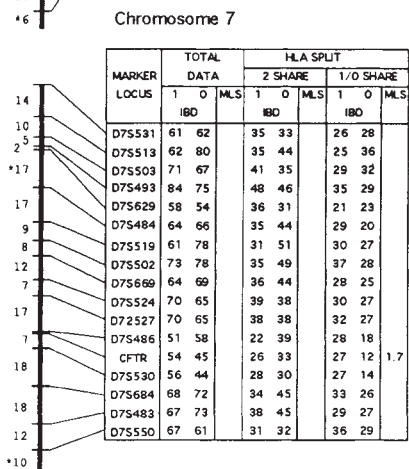
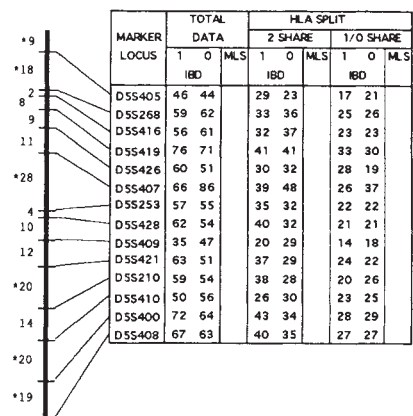
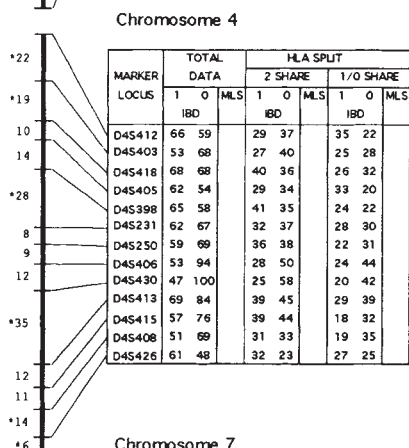
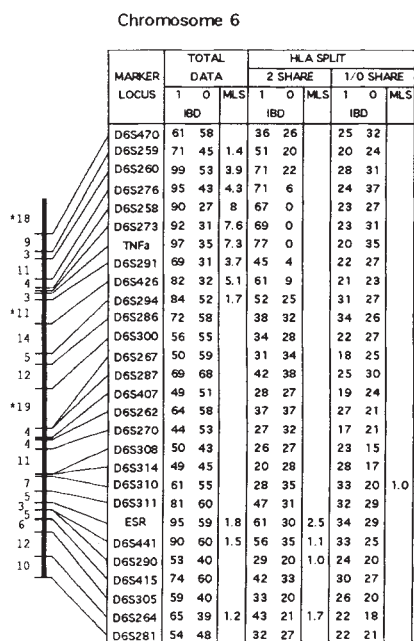
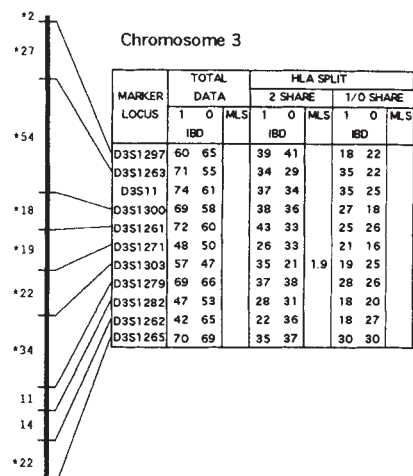
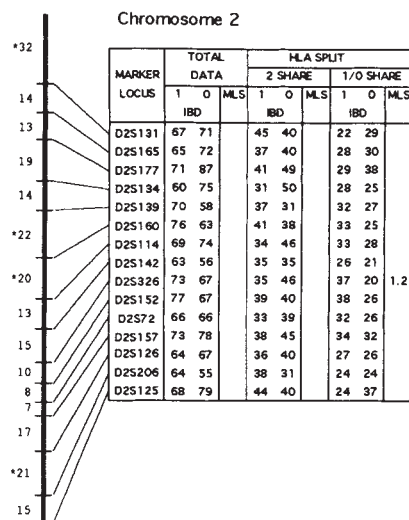
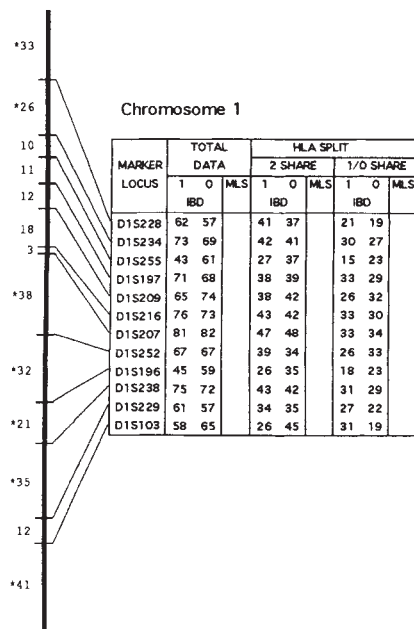
IDDM2 on chromosome 11p15 also showed evidence of linkage to type 1 diabetes (*TH/INS*; $MLS = 2.1$; $\lambda_s = 1.7$; Fig. 1). This MLS value of 2.1 in the UK 96 families is the strongest evidence to date for linkage of *IDDM2* to type 1 diabetes, without having to rely on intrafamilial association analyses or on excluding families with parents homozygous for the *INS* minisatellite locus⁷⁻¹¹.

Eighteen other chromosome regions showed evidence of linkage to type 1 diabetes in the UK 96 families, with $MLS \geq 1.0$ ($P < 0.05$): chromosomes 2q, 3q, 6q (three distinct regions), 7q, 8q, 10cen, 10q, 11q, 13q, 14q, 16q, 17p, 18q, 19q and X (two regions) (Fig. 1). Ten of these exceeded MLS value of 1.7 ($P < 0.005$): chromosomes 3q (*D3S1303*), 6q (*ESR* and *D6S264*), 7q (*CFTR*), 8q (*D8S556*), 10cen (*D10S193*), 11q (*FGF3*), 13q (*D13S158*) and X (*DXS991* and *DXS999*). Chromosomes 6q and 11q were analysed further and are

TABLE 1 Mapping of *IDDM4* and *IDDM5*

Chr	Families	Locus	θ (%)	Total		MLS	HLA 2		MLS	HLA 1,0		MLS
				1	0		1	0		1	0	
6p	UK 96	<i>TNFA</i>		97	35	7.3						
	UK 102			87	36	5.5						
	USA 84			82	27	6.7						
	Total			268	98	19.3						
11p15	UK 96	<i>TH/INS</i>		107	67	2.1	63	40	1.3	44	27	1.0
	UK 102			103	89	0.4	56	45	0.3	47	40	0.2
	USA 84			77	71	0.1	50	38	0.6	26	32	0.0
	Total			287	227	1.6	169	123	1.9	117	99	0.7
11q	UK 96	<i>D11S903</i>		59	66	0.0	33	36	0.0	25	29	0.0
		<i>FGF3</i>	18	71	60	0.2	33	37	0.0	38	21	1.8
		<i>D11S916</i>	4	65	78	0.0	30	47	0.0	34	27	0.4
	UK 102	<i>D11S903</i>		80	55	1.0	37	24	0.6	38	25	0.6
		<i>FGF3</i>	18	70	64	0.2	28	30	0.0	37	28	1.0
		<i>D11S916</i>	4	77	76	0.0	34	34	0.0	40	31	0.3
	USA 84	<i>D11S903</i>		60	48	0.3	29	31	0.0	26	17	0.3
		<i>FGF3</i>	18	67	51	1.1	37	28	1.0	23	18	0.2
		<i>D11S916</i>	4	78	57	1.1	40	30	0.9	29	23	0.2
	Total	<i>D11S903</i>		199	169	0.6	99	91	0.1	89	71	0.4
		<i>FGF3</i>	18	208	175	1.3	98	95	0.0	98	67	2.8
		<i>D11S916</i>	4	220	211	0.2	104	111	0.0	103	81	0.9
	6q	<i>ESR</i>		95	59	1.8	61	30	2.5	34	29	0.1
		<i>D6S290*</i>	3	102	69	1.4	63	36	1.4	39	33	0.1
		<i>D6S305</i>	11	59	40	0.8	33	20	0.8	26	20	0.3
	UK 102	<i>ESR</i>		65	73	0.0	30	29	0.0	27	37	0.0
		<i>D6S290*</i>	3	72	76	0.0	31	33	0.0	30	34	0.0
		<i>D6S305</i>	11	77	62	0.4	35	31	0.1	30	28	0.2
	USA 84	<i>ESR</i>		67	48	1.0	32	27	0.3	27	17	0.8
		<i>D6S290*</i>	3	75	54	1.5	41	30	1.2	28	20	0.7
		<i>D6S305</i>	11	45	58	0.0	23	30	0.0	20	22	0.2
	Total	<i>ESR</i>		227	180	1.5	123	86	1.6	88	83	0.1
		<i>D6S290*</i>	3	249	199	1.5	135	99	2.0	97	87	0.2
		<i>D6S305</i>	11	181	160	0.8	91	81	0.2	76	70	0.7

Linkage of marker loci on chromosomes 6p (*TNFA*), 11p15 (*TH/INS*), 11q (*FGF3* region) and 6q (*ESR* region) to type 1 diabetes in 96 UK families, 102 additional UK families and 84 USA families. Sibpairs were subdivided into those sharing two alleles IBD at *HLA* (*HLA* 2) and those sharing 1 or 0 alleles IBD at *HLA* (*HLA* 1,0). Values of θ were calculated using LINKAGE on the data obtained from these families. *D6S290* was made more informative by haplotyping (denoted by an asterisk) with the flanking marker loci *ESR* and *D6S305*. It has been suggested that the critical level of significance should be increased by $\log_{10}(t)$, where t is the number of tests⁴⁵ (for example, the critical MLS value for stage 1 would be $2.3 + \log_{10}(2) = 2.6$). But, in the case for results which are positively correlated, as for our data, this adjustment may be too conservative, and a number smaller than $\log_{10}(t)$, which remains to be determined, should be added⁴⁵. In the absence of clear theoretical guidelines, we have chosen an unadjusted threshold value for significant evidence of linkage of $MLS \geq 2.3$ (ref. 16), in addition to requiring that the initial linkage was replicated in at least one data set. DNA samples from UK and USA families are available from the BDA and the HBDI, respectively.



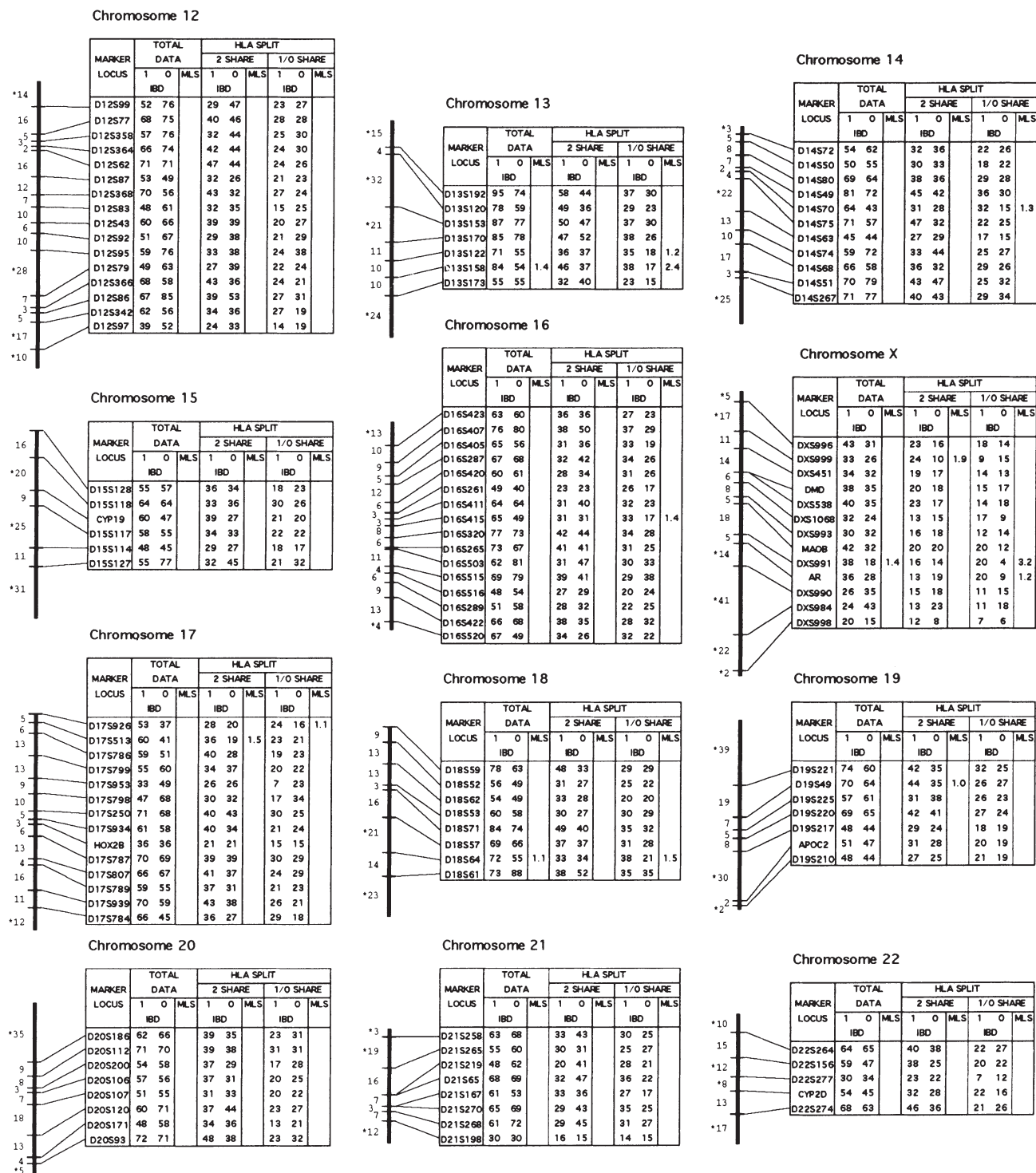


FIG. 1 Genome-wide search for type 1 diabetes susceptibility genes by linkage analysis of 290 marker loci in 96 UK affected sibpair families. The data for sharing 1 or 0 alleles IBD, summed over maternal and paternal meioses, are shown at each microsatellite locus; families were subdivided according to their IBD status at *HLA* (sibpairs sharing two alleles at *HLA* IBD:HLA SPLIT 2 share, and sibpairs sharing 1 or 0 alleles at *HLA* IBD:HLA SPLIT 1/0 share). MLS values were based on the 2,1,0 sharing of alleles IBD at each marker and are shown for values ≥ 1.0 ($P < 0.05$). As described previously¹⁵, families in which the IBD status could not be determined unambiguously (scored as 0 or 1 sharers, 1 or 2 sharers, and 0 or 2 sharers) also contributed to the MLS values. MLS values for the X chromosome were calculated according to the sex of the sibpair and exact significance levels for these remain to be calculated by simulation studies. The linkage map was constructed using genotyping data from the UK 96 families and fluorescence-based chromosome-specific microsatellite sets²³. Interlocus distances are shown as sex-averaged, % recombination fractions (θ) (except for

$\theta \leq 0.01$) when the evidence for linkage, by two-point analysis using LINKAGE, exceeded Lod 3. For the locus-proterminus regions and where the evidence for linkage between two markers was less than Lod 3, the value of θ (denoted by an asterisk) was taken from other maps^{20,46-48}. The most likely position for *D3S11* at 3p14-p21 (ref. 49) is between *D3S1263* and *D3S1300*. The marker locus *TH/INS* was a combination of the marker *TH*, a tetranucleotide microsatellite in the gene encoding tyrosine hydroxylase⁵⁰, 10 kb from the *INS* structural gene, and the *INS* minisatellite locus⁶, which was typed using standard blotting techniques⁶. Results files for all families showing double recombinations in regions of $\theta \leq 0.3$ were re-checked. The accuracy of fluorescence-based genotyping²² was confirmed by comparison with genotypes generated using conventional methods at *HLA* and at *INS*, by typing the *FGF3* microsatellite locus twice using two different sets of primers (two errors in 384 genotypes), and by the fact that our linkage map agrees closely with that of G  n  thon.

reported here. The others are currently being followed up by analysis of additional families and markers.

Stage 2: Mapping of *IDDM4* and *IDDM5*

The *IDDM1*-linked *TNFA* locus showed strong evidence of linkage to type 1 diabetes in all three data sets (UK 96, UK 102 and USA 84; Table 1). However, *IDDM2* (*TH/INS* locus) did not show significant linkage to disease in either the UK 102 families (MLS=0.4; $\lambda_s = 1.0$) or in USA 84 families (MLS=0.1; $\lambda_s = 1.2$; Table 1).

In all families combined (UK 96, UK 102 and USA 84), *FGF3* on chromosome 11q was linked to disease (MLS=1.3; Table 1). However, the most significant evidence of linkage was obtained in the subset of families sharing 1 or 0 alleles IBD at *HLA* (MLS=2.8; $P < 0.001$; $\lambda_s = 1.2$; Table 1). Six additional polymorphic microsatellite marker loci in the *FGF3* region were analysed, giving a peak MLS of 3.4 at *FGF3* ($P < 0.0001$; $\lambda_s = 1.3$; Fig. 2, and data not shown). A disease locus, designated *IDDM4*, may lie in a 14-cM region, between *D11S1253* and *D11S1314* (Fig. 2).

In the UK 96 families, the MLS for the chromosome 6q marker locus *ESR* was 1.8 ($P < 0.005$), and MLS=2.5 ($P < 0.001$) was obtained in the families sharing two alleles IBD at *HLA* (Fig. 1). Linkage of chromosome 6q to disease was also obtained in the USA families (MLS values for *ESR* and *D6S290* were 1.0 and 1.5, respectively; Table 1), thereby replicating the initial finding. There was no support, however, for linkage in the UK 102 families (MLS=0; Table 1). The combined results from all data sets provided strong, albeit not significant support for a disease locus in this region (*D6S290* MLS=2.0; $P < 0.005$ in the category sharing two HLA alleles; Table 1). Nevertheless, given that we did obtain an MLS ≥ 2.3 in the initial data set of 96 UK families, and then obtained further support for linkage in the independent data set of 84 USA families, the results strongly implicate the involvement of this region of chromosome 6q in type 1 diabetes. The locus has been designated *IDDM5*.

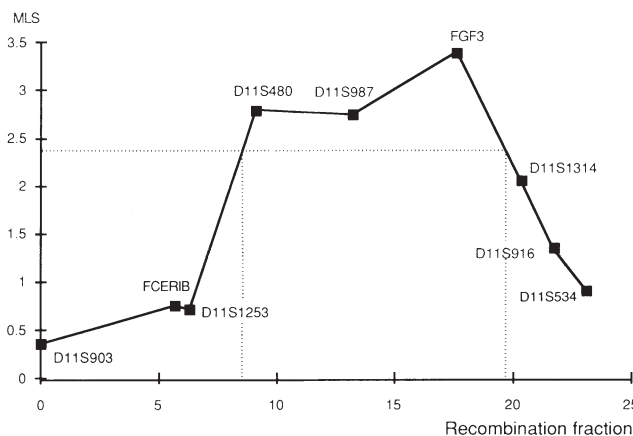


FIG. 2 Location of *IDDM4* on chromosome 11q. MLS values were obtained by analysis of all families (UK 96, UK 102 and USA 84) with both siblings diagnosed under the age of 17 years and sharing *HLA* 1,0 alleles IBD (96 sibpairs). 88%, 75% and 87% of the UK 96, UK 102 and USA 84 families, respectively, had both siblings diagnosed under age 17 years. Dotted lines indicate the one MLS unit support interval for *IDDM4*. The informativeness of the marker loci was increased by haplotyping, which made *FCER1B* fully informative in 70% of the families, *D11S1253* 88%, *D11S480* 76%, *D11S987* 84%, *FGF3* 87%, *D11S1314* 86%, and *D11S916* 88%. The flanking markers *D11S903* and *D11S534* were fully informative in 68 and 79% of families, respectively. MLS values were calculated from the haplotyped data. This approach is simple and in fully genotyped families provides at least as much power as multipoint methods based on joint likelihood calculations. Values of θ were calculated based on genotyping data from all the diabetic families using the LINKAGE program.

TABLE 2 Linkage of *IDDM4* and *IDDM5* by HLA-DR type

Families	Locus	DR3/4		MLS	DR3		MLS
		1	0		1	0	
UK 96	<i>FGF3</i>	30	34	0.0	15	14	0.0
UK 102		38	40	0.0	22	14	0.4
USA 84		38	16	2.2	13	8	0.3
Total		106	90	0.6	50	36	0.6
UK 96	<i>ESR</i>	39	17	1.8	16	8	0.6
UK 102		25	28	0.0	13	12	0.1
USA 84		25	14	0.7	13	2	2.1
Total		89	59	1.3	42	22	1.6
Families	Locus	DR4		MLS	Others		MLS
		1	0		1	0	
UK 96	<i>FGF3</i>	35	34	0.1	10	3	0.7
UK 102		33	29	0.4	7	5	0.1
UK 84		30	36	0.0	8	4	0.6
Total		98	99	0.2	25	12	0.6
UK 96	<i>ESR</i>	33	28	0.1	7	6	0.0
UK 102		21	27	0.0	4	6	0.0
UK 84		25	28	0.0	4	4	0.0
Total		79	83	0.0	15	16	0.0

The 1,0 IBD sharing data at the *IDDM4*-linked locus *FGF3* and the *IDDM5*-linked locus *ESR* in affected sibpairs according to HLA-DR type are shown. In the HLA-DR3/4 category both siblings were DR3/4 (identical-by-state). In the DR3 category both siblings were DR3-positive; sibpairs in which both siblings were DR3/4 heterozygotes, and sibpairs in which both siblings were DR4-positive were not included in this category. Similarly, the DR4 category contained only those sibpairs in which both siblings were DR4-positive but not DR3/4. The Others category contained the remaining sibpairs. MLS values were calculated as described (ref. 15, Fig. 1 legend).

Linkage of *IDDM4* and of *IDDM5* to disease was analysed according to HLA-DR types of the sibpairs (Table 2). In all three family data sets combined, there was no significant evidence of heterogeneity ($P > 0.05$; evaluated by χ^2 contingency table analysis) in the linkage of *FGF3* or *ESR* to disease by HLA type. However, there was heterogeneity at *IDDM4*-linked locus *FGF3* by HLA type in the USA families ($\chi^2 = 8.17$; 3 d.f.; $P = 0.04$), with *FGF3* showing the strongest evidence of linkage in the sibpairs who were both HLA-DR3/4 (MLS=2.2; $P < 0.005$). These data indicate that the linkage of chromosome 11q to type 1 diabetes differs between USA and UK families in the HLA-DR3/4 category ($\chi^2 = 8.0$; 2 d.f.; $P = 0.02$), and, at least in USA sibpairs, according to their HLA-DR status. At the *IDDM5*-linked locus *ESR*, there was also evidence for heterogeneity between family data sets in the HLA-DR3/4 category ($\chi^2 = 6.08$; 2 d.f.; $P = 0.05$), and the USA families according to HLA type ($\chi^2 = 8.43$, 3 d.f.; $P = 0.04$), with the strongest evidence of linkage in the sibpairs who were both HLA-DR3 positive (MLS=2.1; $P < 0.005$).

Discussion

We have searched the human genome for type 1 diabetes susceptibility genes using 96 affected sibpairs and a linkage map of 290 marker loci (Fig. 1). The fluorescence-based genome linkage map had an average spacing of 11 cM (35 additional microsatellites were added to the index map described previously²³) and the marker loci had a mean heterozygosity of 0.8. Although gaps exist in the map, particularly at proterminal regions, only about 3% of the genome remains to be covered at a 20-cM resolution. Simulation analyses revealed that there is a 99.9% probability of obtaining MLS ≥ 2.3 for a locus with $\lambda_s = 2.5$ in 96 affected sibpair families using these markers. It seems unlikely, therefore, that another locus with an effect equivalent to *IDDM1* exists. For a locus with an effect equivalent to *IDDM2* ($\lambda_s = 1.7$ in UK 96), there is a 46% probability of obtaining MLS ≥ 1.0 , and a 12% probability of obtaining MLS ≥ 2.3 . Thus, we have prob-

ably not detected all of the disease loci in the $\lambda_s = 1.2$ –1.8 range. It is also noted that a marker locus with heterozygosity of 0.8 situated only 3 cM from *IDDM2* would not have shown $MLS \geq 1.0$ in the UK 96 families. Therefore, to identify all the major genes for type 1 diabetes by linkage analysis, a map with markers at 3-cM intervals will be required, as well as several hundred sibpairs. These considerations highlight the advantages of incorporating intrafamilial association studies¹¹ (or well designed case-control studies³¹) and candidate gene analyses into searches for genes influencing complex traits.

Owing to the failure to replicate linkage data in psychiatric diseases^{31,34,35}, confirmation of the initial findings is essential as it is extremely unlikely that false-positive results would be replicated in additional data sets. In the present study, therefore, three different sets of sibpair families, one of which, from the USA, was collected independently, have been analysed to verify linkages on chromosomes 6q and 11q. Support for linkage of *FGF3* and flanking markers on chromosome 11q to type 1 diabetes was found in the UK 96, UK 102 and USA 84 families ($MLS = 3.4$; $P < 0.0001$, *IDDM4*; Table 1 and Fig. 2). Evidence of linkage to chromosome 6q near the marker loci *ESR* and *D6S290* was found in the UK 96 families and also in the USA 84 families ($MLS = 2.0$; $P < 0.005$, *IDDM5*; Table 1). We did not find linkage of disease to *IDDM5* in the UK 102 families, nor was *IDDM2* linked to disease in the UK 102 and USA 84 families, even though the mapping of *IDDM2* in type 1 diabetes is well established by both intrafamilial association studies^{7,8,11} and linkage analyses^{9,10}. Problems with ascertainment, diagnostic criteria, aetiological heterogeneity and failure to correct for the testing of multiple genetic models, have all been blamed for failure to replicate linkages in other family data sets^{34,35}. It has been shown³⁴ by computer simulation that if a complex trait is caused by, for example, six loci with equal effects, then the number of families required to achieve replication is approximately five times the number of families required for the first detection ($N-1$, where N is the number of loci). This may help explain why we have failed to replicate the results for *IDDM2* and *IDDM5* in all three data sets.

IDDM4 on chromosome 11q would not have been detected if we had not evaluated linkage of markers to disease simultaneously with linkage to *IDDM1*. Linkage of chromosome 11q to type 1 diabetes was probably not detected previously³⁶ because this approach was not taken. More detailed localization of *IDDM4* within the 14-cM region of chromosome 11q (Fig. 2) will require the analysis of additional microsatellite loci and other polymorphisms by testing for associations of marker alleles with disease in families. This should be feasible, given the current and improving status of the genetic²⁰ and physical³⁷ maps, and the advantages of the fluorescence-based technology²³.

How many of the 18 MLS values ≥ 1.0 represent true effects (ignoring the additional data for *ESR* and *FGF3*) and how many are false positives due to chance (ref. 33)? The proportion of potential false positives can be estimated from our own data set. During stage 1 (Fig. 1) it was noted that at nine unlinked marker loci more sibpairs shared zero alleles IBD than one allele ($P < 0.05$, or negative quasi-MLS ≥ 1.0 ; Fig. 3). As these negative distortions have no plausible biological meaning they must represent random events. Only one negative sharing locus, *D4S430*, exceeded a quasi-MLS value of 1.8 ($P < 0.005$) in the UK 96 families. This, as expected, was not replicated in the UK 102 or USA 84 families (Fig. 3 legend). In contrast, there were seven loci with positive MLS values > 1.8 . These data suggest that many of the loci with positive MLS values less than 1.8 may be false, whereas the majority of loci with MLS values > 1.8 are probably indicative of genuine susceptibility genes.

Some of the linked marker loci occur in regions that contain candidate genes. Notably, *D2S326* and *D10S193* are near the *GAD1* and *GAD2* genes, respectively, which encode the enzyme glutamic acid decarboxylase, a pancreatic β -cell autoantigen in type 1 diabetes. *ESR* on chromosome 6q maps close to the gene

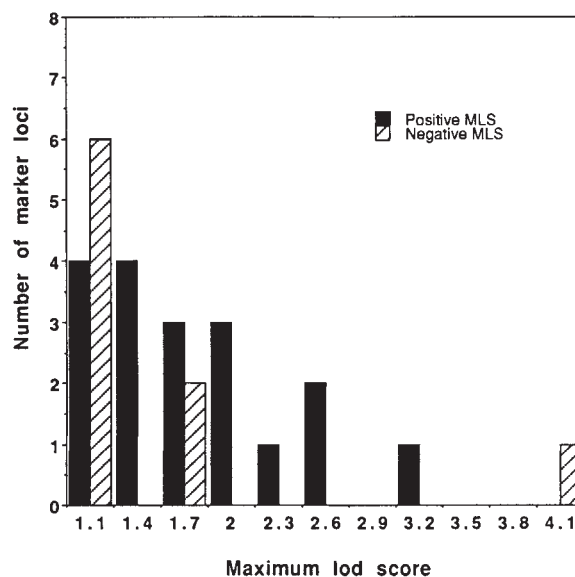


FIG. 3 Distributions of MLS values for marker loci showing positive (filled bars) and negative (hatched bars) quasi-MLS values ≥ 1.0 in the UK 96 families (data from Fig. 1). The negative quasi-MLS values for the nine marker loci showing more 0 sharing than 1 sharing IBD, were estimated by dividing the χ^2 values by $2 \times \ln 10$. χ^2 values were calculated from the 1,0 distributions given in Fig. 1 against an expected distribution of 1:1. The nine marker loci were *D1S103*, *D3S1262*, *D4S408*, *D4S430*, *D7S486*, *D7S519*, *D8S258*, *D10S189* and *D21S219*. In Fig. 1 MLS values for these markers were 0.0 because they exceeded the boundaries of the possible sharing probabilities. *D4S430*, which had a negative quasi-MLS of 4.1 in the UK 96 families, was analysed in the UK 102 and the USA 84 families, and, as predicted for a chance event, no distortions of the sharing of alleles IBD were observed (the 1,0 sharing distributions for the UK 102 and USA 84 families were, 83,79, and 67,70, respectively).

SOD2, encoding superoxide dismutase, which may relate to the sensitivity of β -cells to oxygen free radical damage. A restriction-fragment length polymorphism of the *SOD2* gene has been associated with type 1 diabetes³⁸. Two regions that showed linkage to disease, on chromosomes 2q and 17p, possibly contain the homologues of the mouse insulin-dependent diabetes genes, *Idd5* and *Idd4*, respectively^{1,2,39}. The microsatellite locus *D18S64*, which in the UK 96 families showed evidence of linkage to type 1 diabetes (Fig. 1; $MLS = 1.1$), is in the same region of chromosome 18 as the Kidd blood group locus⁴⁰. Linkage to Kidd blood group was obtained over 10 years ago (initially a Lod score of 2.8 was obtained using the LIPID program under several different genetic models)^{41,42}, and, although a second independent study failed to replicate the linkage⁴³, a population-based association between Kidd blood group and type 1 diabetes was reported⁴⁴. Taken together with the linkage we have observed on chromosome 18, the data suggest that a susceptibility gene lies very close to or at the Kidd blood group locus.

The transmission of human type 1 diabetes is clearly complex. Indeed it appears from this study that a proportion of the familial clustering unaccounted for by *IDDM1* and *IDDM2* is caused by at least three other genes. □

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LETTERS TO NATURE

Carbon and the formation of reduced chondrules

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CHONDRULES are millimetre-sized spheroidal bodies composed mainly of olivine and orthopyroxene, which comprise the dominant fraction of most chondritic meteorites. They are the products of partial melting of aggregates of fine-grained silicates with minor contributions from metals, sulphides and oxides. Although the formation conditions of chondrules are not well understood, these are thought to involve a transient melting event in the solar nebula^{1–3}. The ubiquity of reduced carbon in interstellar clouds and primitive meteorites implies that it was also present in the early solar nebula, and may thus have been a potential constituent of chondrule precursor material. We describe here experiments in which carbon and magnesian silicate precursor material of primitive chondrule composition are 'flash-heated' together and then crystallized. The resulting material shows many mineralogical features characteristic of natural chondrules, which are not produced in the absence of carbon^{4–12}. Our results suggest not only that carbon was present in the solar nebula, but also that it played a key role in chondrule formation by creating within the melt a reducing environment that was decoupled from the nebula gas.

Previous dynamic crystallization (nonequilibrium) experiments^{4–12} that have studied chondrule formation assumed that chondrule precursors were only silicates. However, natural chondrule precursors almost certainly were a complex, heterogeneous mixture of silicates and non-silicates. Amongst these non-silicates, reduced carbon in the form of organic or graphitic material was probably among the most abundant and chemically important. It has been suggested¹³ that chondrule precursors may have even been held together with some form of organic

'glue'. The occurrence of carbon in chondrites has been described^{14–17}. Furthermore, the amount of carbon in 'pristine' early Solar System materials such as CI chondrites and the matrix of type 3 ordinary chondrites is up to 5% (ref. 18) and it may be that this is a reasonable amount to expect in the chondrule precursor.

Experiments (Table 1) were performed at 1 atm in an Astro furnace. Two starting materials of the same composition with initial grain sizes of 23–45 µm and 125–250 µm were prepared from ground silicate minerals (70% San Carlos olivine, 10% orthopyroxene, 10% diopside and 10% albite). Graphite (grain size <1 µm to 200 µm) and diamond (grain size 0.5 µm) were added to our silicate starting materials (from 0 to 10% wt%) for a combined pellet weight of 25 mg. Our experiments used graphite and diamond because the highly volatile, explosive nature of many forms of organic carbon prevents its use in performing experiments in our furnace. We are confident the redox properties of carbon in this form will match that of organic material closely enough for relevant conclusions to be drawn concerning the effects of carbon upon mineral chemistry and texture. The oxygen fugacity f_{O_2} was maintained at IW-1.5 with a mixture of CO and CO₂. Thermal conditions imposed on pellets follow techniques described elsewhere^{11,12}.

Experiments Ast 32 and Ast 33 were carbon free and are our baseline (control) charges. Charge Ast 32 has a texture similar to type IA chondrules (Fig. 1a) whereas charge Ast 33 has a porphyritic texture with numerous relict (unmelted) olivines. The compositions of the olivines in these charges are listed in Table 1. These charges also contain rare, inclusion-free, Fe-rich, metal grains <10 µm in size.

Porphyritic textures similar to Ast 32 and Ast 33 were produced from charges with added carbon (Fig. 1b). However, the addition of 1–5% carbon in the starting material produces olivine phenocrysts with increasing MgO (termed forsterite, Fo) contents (Figs 1b, 2a, b). Adding >5% carbon to the starting material results in little further change in melt-grown olivine composition.

Relict olivines in charges with added carbon contain numerous metal grains, on average <1 µm in diameter (Fig. 1c). Microprobe analysis of the largest metal grains in olivines determined that they are kamacite (Fe-Ni with variable, low concentration of Ni) metal with variable amounts of Ni. Other relict olivines have veins of Fe metal and veins of more MgO-rich olivine (Fig. 1d). These olivines are similar to natural dusty olivines^{19,20} that have been interpreted as surviving chondrule precursor sol-

TABLE 1 Experimental details and results

Charge number	Type of carbon				Amount of carbon (wt%)			Grain size of silicates (μm)			Average Fa (mol%)
Ast 32	None				0			23–45			5.93
Ast 33	None				0			125–250			6.48
Ast 70	Diamond				1			23–45			4.75
Ast 69	Diamond				3			23–45			4.45
Ast 56	Diamond				5			23–45			3.37
Ast 48	Diamond				5			23–45			2.8
Ast 76	Graphite				1			125–250			3.59
Ast 67	Graphite				1			23–45			3.61
Ast 75	Graphite				3			125–250			1.62
Ast 45	Graphite				3			23–45			2.18
Ast 46	Graphite				3			125–250			0.89
Ast 38	Graphite				5			23–45			0.62
Ast 68	Graphite				7			23–45			0.61
Ast 59	Graphite				10			23–45			0.36
Name	SiO ₂	TiO ₂	Al ₂ O ₃	FeO	MnO	MgO	CaO	Na ₂ O	K ₂ O	Total	Calculated liquidus temperature
Type IA	46.42	0.08	3.09	7.92	0.12	38.23	2.51	1.32	0.05	99.74	1,692 °C

This Table lists experimental charge numbers, the type of carbon used, the amount of carbon added to our type IA chondrule composition, the initial grain size of our type IA composition and the average final Fa (FeO-rich olivine) content of olivine grains without visible relict grains. The last line is an analysis of the bulk composition of our starting material, type IA.

ids. Dusty olivines were argued to have been reduced within their host chondrule during chondrule formation by the loss of an oxidized phase such as H₂O or CO (ref. 20). Such reduction might have been achieved by either the surrounding nebular H₂ reacting with the melted chondrules or by the presence of carbon within the precursors. Previous experiments²¹ have not been able to produce dusty olivines in reducing furnace gases, thus provid-

ing additional support for carbon as a reducing agent in chondrule precursors.

Charges with added carbon also exhibit numerous metal grains dispersed throughout. Depending on their sizes (and probably on the amount of local silicate reduction needed to produce them) these metal grains exhibit very different compositions. Numerous, large, Fe-rich, metal grains (up to several hun-

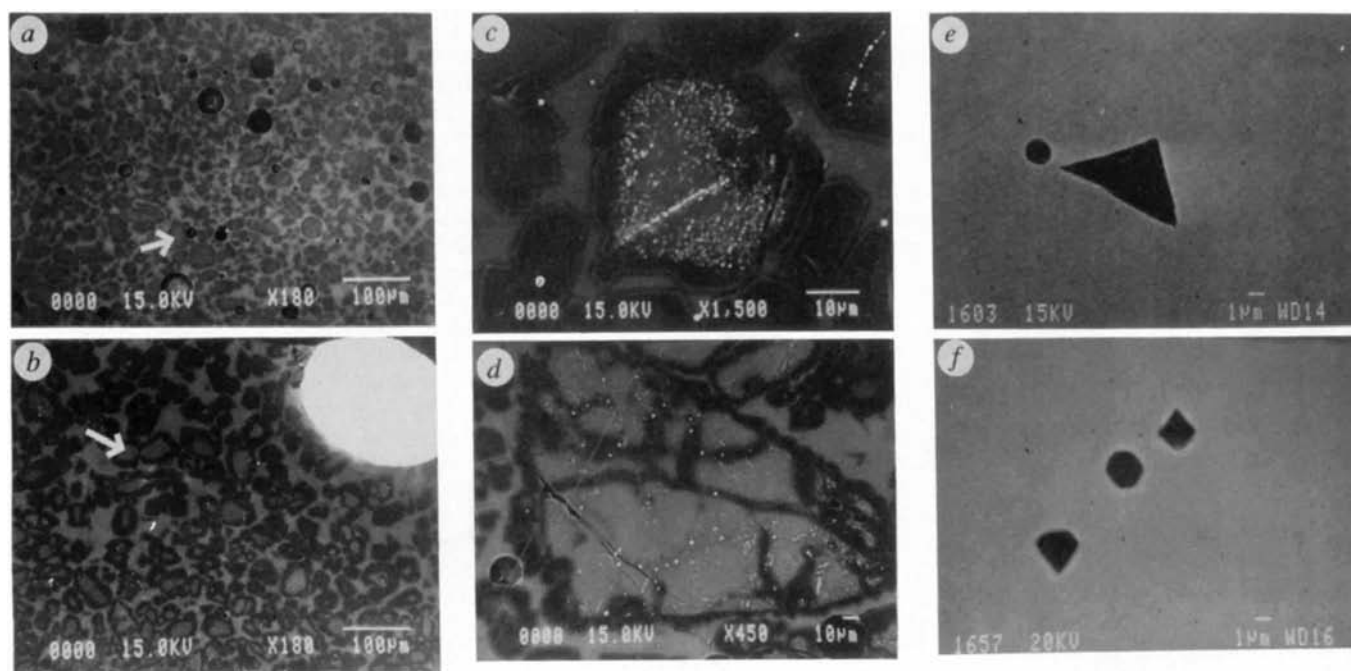
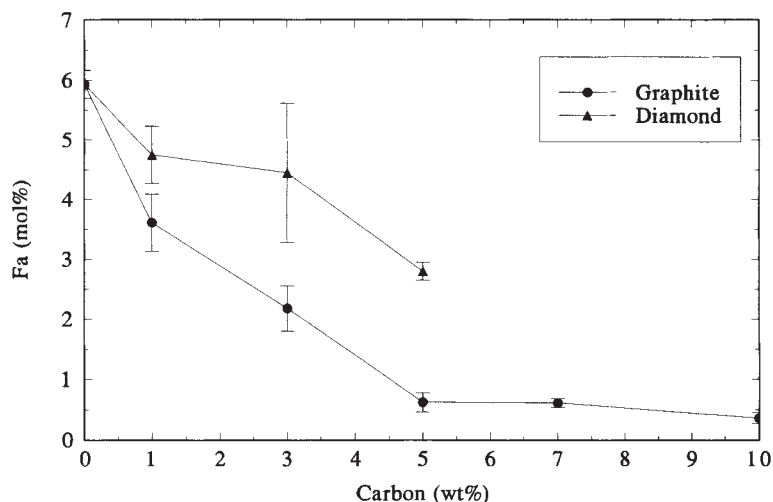


FIG. 1 a, A backscatter electron image from a charge with no added carbon (Ast 32). Arrow points to light-grey core of an olivine. These light-grey cores are unmelted or relict olivines that are overgrown by new olivine (darker grey). The image shows no metal grains as experiments without added carbon are virtually free of metal. b, Backscatter electron image from charge with 7% added carbon (Ast 68). The arrow points to a relict, dusty olivine that is overgrown by much more Mg-rich olivine (dark grey). The overgrowths in this charge are much darker in colour, indicating a much greater Mg content than overgrowths seen in Ast 32. The large, bright white grain in the right corner is a large metal (kamacite) grain. The metals in our experiments appear as bright white in these images. Lots of smaller, metal (taenite) grains can be seen in the lower left part of the image. c, Backscatter electron image from charge Ast 68

showing a synthetic, relict, dusty olivine. The white-coloured spots are Fe and Fe–Ni-rich metal grains. d, Backscatter electron image from charge Ast 76 showing another type of synthetic, relict, dusty olivine grain. The darker colour along cracks in the grain is Mg-rich olivine, indicating that this relict grain reacted with the reduced melt. e, Backscatter electron image from charge Ast 68 showing Si-bearing inclusions in a kamacite grain. Euhedral inclusion is pure SiO₂ whereas spherical inclusion is fayalitic (Fe-rich olivine) glass. f, Backscatter electron image from a chondrule in the meteorite Acfer 207, showing euhedral and subhedral SiO₂ inclusions with an Fe-rich metal grain. Such inclusions are found throughout our synthetic chondrules. Scale bars; a, b, 100 μm ; c, d, 10 μm ; e, f, 1 μm .

FIG. 2 Plot of the average final Fa (FeO-rich olivine) content of melt-grown olivines against the amount of initial carbon in the starting material; the initial grain size of the starting material was 23–45 μm . Use of the alternative-grain-sized starting material (125–250 μm) does not significantly affect this plot.



dreds of micrometres in size) were found on the surfaces (bottoms, tops and sides) and in the interior, often near the internal edges, or charges. These large grains typically contain only 1–2 wt% Ni and ~ 0.2 wt% Co, and have significant amounts of dissolved Cr and Si (usually a few tenths of a weight per cent). Such metal compositions are similar to those of metal grains found in natural, reduced chondrules from highly unequilibrated chondrites^{22–24}. In addition, the large metal grains also contain silica and fayalitic glass inclusions that strikingly resemble those described in chondritic metal (refs 24, 25 and Fig. 1e, f). We believe that these grains formed early in the melting process when charges were mostly molten and much of the carbon had oxidized to produce a locally reducing environment. Large grains located at and near the charges' edges form by coalescence of smaller grains that migrate freely by surface tension. The smaller metal grains in our charges are taenite (high concentrations of Ni) with 25–30 wt% Ni, >1 wt% Co with neither dissolved Cr and Si nor detectable inclusions. These metal grains were probably formed during crystallization: once crystallization begins, metal grains become trapped by growing crystals and cannot coalesce or migrate as easily. In general, experiments²¹ with reduction from external gases produced metal mainly on the surface of charges and not throughout like our experiments. Therefore, chondrule metal was probably produced by reduction induced by an internal agent, such as carbon.

In addition to metal, charges with 7% and 10% added graphite also had graphite grains ($<100 \mu\text{m}$) on their surfaces often associated with tiny metal and SiO_2 grains ($<5 \mu\text{m}$). The existence of graphite grains suggests that such large amounts of added graphite cannot totally oxidize during the imposed heating cycle. In the primitive chondrite Bishunpur, graphite occurs in metal at the periphery of chondrules and in matrix metal that is poor in Cr and Si (refs 15, 26). Metal in chondrules is usually higher in dissolved Cr and Si comparatively and so are larger matrix grains with no graphite inclusions. Large matrix metal grains were probably lost from chondrules²⁴. The presence of graphite in metal that is poor in Cr and Si has been interpreted²⁶ as an indication that this metal was lost from chondrules before extensive reaction (or reduction) occurred (that is, early in the chondrule-forming process).

It is clear that the charges with carbon added to the starting material have experienced formational conditions that were far more reducing than the charges without carbon added to the starting material, independent of the furnace gases. Graphite generates olivines more MgO-rich than did our experiments with

diamond (Fig. 2a). The reason for this difference may be that the finer-grained diamond is oxidized much more quickly than the graphite, permitting the furnace gases to interact with the charge earlier in the cooling cycle to produce charges that approach (but do not reach) equilibrium with the furnace gases sooner than the graphite experiments. Additionally, experiments with $>5\%$ added carbon produce little further reduction in the olivine compositions. If melting was more extensive, more of the unmelted olivine would melt to provide additional material for reduction, hence more MgO-rich olivine compositions and fewer dusty olivines.

By adding carbon to the starting material we have created a microenvironment within and around our charges in which the carbon reacts with the oxygen from the melt and also the solid phases (dusty olivines), thus reducing the charges independently of the furnace gases. If carbon did play an important role in forming reduced chondrules, as our results suggest, then caution must be shown when inferring the f_{O_2} of the nebular gas from chondrules. Calculations based on Fe– SiO_2 –Fe $_2\text{SiO}_4$ equilibria²⁷ yield $\log f_{\text{O}_2} \approx -12$ at 1,600 $^\circ\text{C}$ for charges like Ast 68 (Table 1, Fig. 1c) as well as for natural chondrules with similar olivine compositions²⁸. The $\log f_{\text{O}_2}$ of the ambient 1 atm furnace gas was -9.5 and the canonical solar nebular was -14.7 at this temperature. Our experimental chondrules were internally buffered by carbon, as would be natural chondrules with carbon in the precursors. In general, chondrules are unlikely to have reached redox equilibrium with nebular gas during rapid heating and cooling²⁹; it is more likely that redox conditions in the chondrule were buffered by precursor minerals and not determined by nebular conditions. $\square$

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Probable hole-doped superconductivity without apical oxygens in $(\text{Ca}, \text{Na})_2\text{CuO}_2\text{Cl}_2$

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THE presence of apical oxygens (above or below the CuO_2 planes) has been regarded as indispensable for the occurrence of hole-doped superconductivity in the high-transition-temperature (high- T_c) copper oxide superconductors^{1,2}. We have opposed this view^{3,4}, on the basis of studies in compounds such as $(\text{Ca}, \text{Sr})_{1-x}\text{CuO}_{2-y}$ and $\text{Sr}_{n+1}\text{Cu}_n\text{O}_{2n+1+\delta}$; however, our conclusions have been questioned in subsequent work^{5,6}. Here we provide strong evidence for hole-doped superconductivity in the absence of apical oxygens, by showing that $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ can be rendered superconducting by doping with sodium. The compound contains CuO_2 planes as in La_2CuO_4 , but all of the oxygen atoms at the apices of the CuO_6 octahedra found in La_2CuO_4 are replaced by chlorine, and the lanthanum atoms by calcium⁷. The apparent difference in mass and electronic character between chlorine and oxygen might strongly affect the electronic structure of the CuO_2 planes if the contributions of the apical atoms were significant. But we find hole-doped superconductivity in $(\text{Ca}, \text{Na})_2\text{CuO}_2\text{Cl}_2$ at rather high transition temperatures (26 K at maximum), which are only slightly less than those of doped La_2CuO_4 (ref. 8). This implies that the apical atoms do not play a significant role in the electron pairing mechanism leading to high- T_c superconductivity.

$\text{A}_2\text{CuO}_2\text{Cl}_2$ (where A stands for Ca or Sr) has been proposed as a candidate for high- T_c superconductivity, because it is essentially isostructural with the most well-known superconductor $(\text{La}, \text{Sr})_2\text{CuO}_4$. The physical properties of the undoped CuO_2 planes are quite similar in the $\text{A}_2\text{CuO}_2\text{Cl}_2$ compounds⁹, but so far they have not shown evidence of superconductivity. There are two possible reasons for this. First, chemical modification by doping may destabilize the crystal structure. Alternatively, the absence of apical oxygen atoms may destroy the superconducting ground state of the CuO_2 planes. We have synthesized these compounds in order to investigate these possibilities. Recently, Al-Mamouri *et al.*¹⁰ reported the observation of superconductivity in $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ at 46 K using samples prepared by low-temperature fluorination of Sr_2CuO_3 . In their compounds, apical oxygen atoms seem to be completely replaced by fluorine atoms.

Recent studies¹¹ have shown that chemical modifications of the copper oxides that are not possible at ambient pressure can in some cases be realized at high pressure. Using such a high-pressure synthetic route, we have succeeded in doping $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ with sodium. The base material $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ was prepared from CaCl_2 , CaO and CuO . The powder mixture was pressed into a pellet and calcined at 1,073 K in a nitrogen flow. The resultant product was single-phase, as determined by powder X-ray diffraction. In order to introduce hole carriers, substitution of Na^+ for Ca^{2+} was attempted, because their ionic radii are close ($r(\text{Na}^+) = 1.18 \text{ \AA}$; $r(\text{Ca}^{2+}) = 1.12 \text{ \AA}$) (ref. 12). Sodium perchlorate (NaClO_4) was used as a source of sodium and chlorine, and also as a creator of oxidizing atmosphere: NaClO_4 powder of up to 10 wt% and appropriate amounts of CuO powder were mixed thoroughly with $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ in a dry box, and the mixtures (sealed in gold capsules) were heated at 1,173 K and 6 GPa for 30 min, using a cubic-anvil-type high pressure apparatus.

Phase identification was done by means of powder X-ray diffraction. Figure 1 shows typical X-ray diffraction patterns for $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ treated under high pressure with and without NaClO_4 . The X-ray diffraction pattern of $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ remained essentially the same after the high-pressure treatment, whereas a slight change in the lattice constants due to the addition of 5 wt% of NaClO_4 lead to shifts and splittings of the peaks. Small amounts of impurities (calcium chloride hydrate ($\text{CaCl}_2 \cdot \text{Ca}(\text{OH})_2 \cdot \text{H}_2\text{O}$) and CuO) were found, the concentrations of which seemed to increase with increasing NaClO_4 content. The calcium hydrate might have formed in the process of grinding and handling after the synthesis. No trace of NaCl was seen, suggesting that sodium was introduced into the oxychloride. No other impurity phases were detected up to 8 wt% of NaClO_4 , although the present phase decomposed completely at 10 wt%. The high-pressure samples were found to be stable in dry air but not in moist air.

In contrast to the structural similarities, we observed dramatic changes in physical properties between the samples prepared

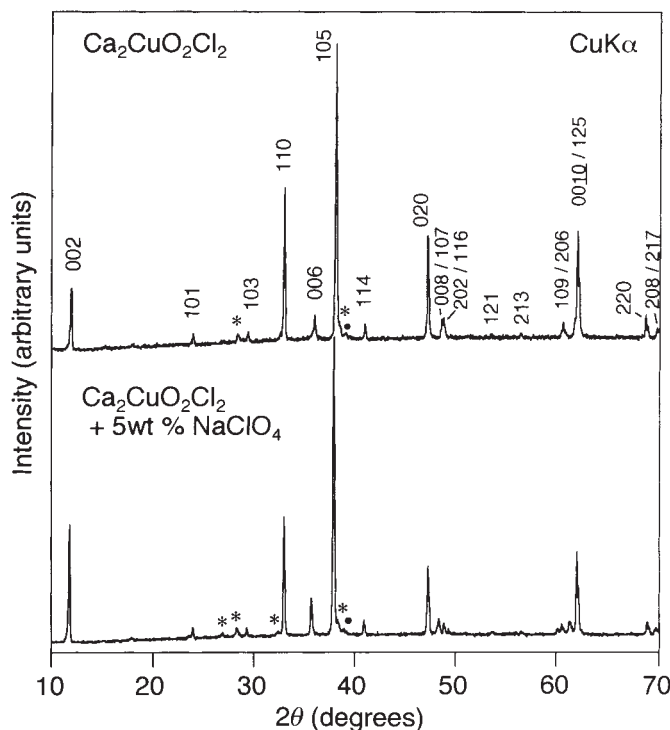


FIG. 1 X-ray diffraction patterns of $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ prepared at high pressure without (top) and with (bottom) 5 wt% NaClO_4 . Several weak peaks are due to impurities (★, $\text{CaCl}_2 \cdot \text{Ca}(\text{OH})_2 \cdot \text{H}_2\text{O}$; ●, CuO).

with and without NaClO_4 : pure $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ was an insulator, whereas samples prepared with NaClO_4 showed much lower resistivity at room temperature and large diamagnetism below a critical temperature. Figure 2 shows the typical temperature dependence of magnetic susceptibility measured with a SQUID magnetometer for a sample treated with 5 wt% NaClO_4 . The distinctive drop at 26 K strongly suggests the occurrence of superconductivity. The transition is rather sharp, and the superconducting volume fraction estimated at 5 K is $\sim 10\%$. The rather small volume fraction is mostly attributed to the small particle size of a few μm as seen in a scanning electron microscope: the total diamagnetic signal should be considerably reduced by the serious penetration of the magnetic field. A much smaller volume fraction of $\sim 1\%$ was observed only for a sample treated with 3 wt% NaClO_4 , probably as a result of an inhomogeneous distribution of the dopant species in addition to the particle-size effect. No change in the susceptibility curve was observed after sample storage of a few weeks at normal conditions. Thus it may be concluded that the major phase of $\text{Ca}_2\text{CuO}_2\text{Cl}_2$, most probably doped with sodium, has really become superconducting. However, a corresponding resistive transition has not yet been measured successfully, mainly because of high contact resistance at the grain boundaries.

The variations of the lattice parameters and T_c are plotted in Fig. 3 against NaClO_4 content. As NaClO_4 content increases, the a -axis length decreases linearly, while the c -axis length increases linearly. This suggests that the chemical doping is accomplished systematically, and thus the carrier concentration in the CuO_2 planes is controlled continuously. Correspondingly, T_c decreases monotonically from 26 to 15 K. As the carrier concentration must be increased with increasing NaClO_4 content, the corresponding decrease in T_c suggests that the present superconductors lie in the over-doped region. The maximum T_c value is slightly lower than that of $(\text{La}, \text{Sr})_2\text{CuO}_4$, but nearly the same as that of $(\text{La}, \text{Ba})_2\text{CuO}_4$ (ref. 8).

The nature of the superconducting carriers cannot be assigned conclusively at present. But the fact that a highly oxidizing atmosphere during the high-pressure treatment is always necessary strongly suggests that the carriers are holes. Moreover, the observed shortening of the a -axis with increasing carrier concentration is in good agreement with the general trend for hole-doped superconductors. There are three possible doping mechanisms. The most probable and major one is partial substitution of Na^+ for Ca^{2+} . It was confirmed by X-ray microanalysis that sodium certainly existed within the crystals, though a quantitative analysis could not be done because of an instrumental

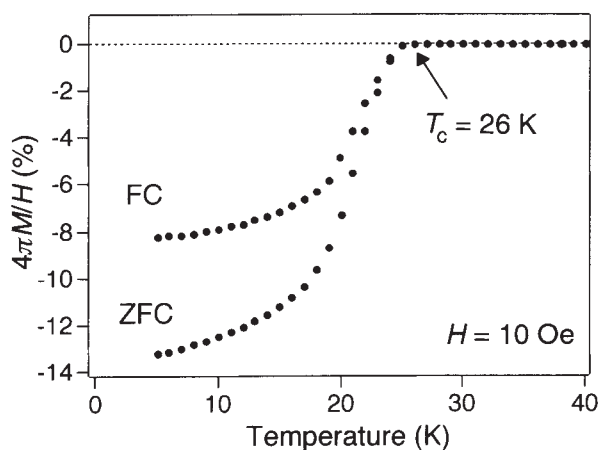


FIG. 2 Temperature dependence of magnetic susceptibility ($4\pi M/H$) for $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ treated under high pressure with 5 wt% NaClO_4 , showing a superconducting transition at $T_c = 26$ K. The ZFC curve was obtained on heating in a magnetic field of 10 Oe after zero-field cooling, and the FC curve was done successively on cooling in the same field.

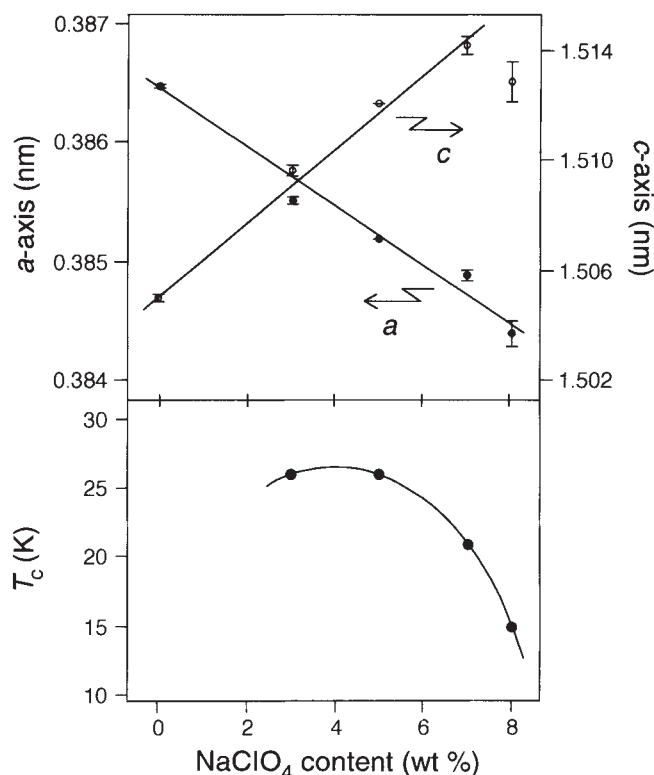


FIG. 3 Variations of lattice parameters (top) and T_c (bottom) as a function of NaClO_4 content.

limit. The important role of sodium can be seen also from the fact that no superconducting signal was detected when we used KClO_4 instead of NaClO_4 . Simply assuming that all the Na^+ ions are situated at the Ca^{2+} sites, 4 wt% of NaClO_4 (around which the maximum T_c is attained; see Fig. 3) gives a composition approximately $(\text{Ca}_{0.96}\text{Na}_{0.04})_2\text{CuO}_2\text{Cl}_2$ which corresponds to 0.08 holes per Cu atom. Because the optimum doping level to attain the maximum T_c is generally considered to be 0.15 holes per Cu, as typically found for $(\text{La}_{0.925}\text{Sr}_{0.075})_2\text{CuO}_4$ (ref. 13), it might be necessary to assume additional doping mechanisms. The second possibility is partial substitution of O^{2-} for Cl^- at the apical site, because NaClO_4 always generates oxygen in the gold capsule. The excess oxygen content necessary to accommodate this loss is 0.035 per formula unit, which means that 3.5% of Cl^- ions are replaced by O^{2-} ions. Apical oxygen atoms are almost entirely absent even in this case. The last possibility is incorporation of interstitial Cl^- ions or O^{2-} ions, as reported for $\text{Sr}_2\text{CuO}_2\text{F}_{2+\delta}$ (ref. 10) and $\text{La}_2\text{CuO}_{4+\delta}$ (ref. 14). We doubt that this is occurring in the present case, because excess oxygen atoms in $\text{La}_2\text{CuO}_{4+\delta}$ are located at interstitial sites coordinated tetrahedrally by the La^{3+} ions, whereas the corresponding sites in $\text{Ca}_2\text{CuO}_2\text{Cl}_2$ seem to be obstructed by large Cl^- ions ($r(\text{Cl}^-) = 1.81 \text{ \AA}$; $r(\text{O}^{2-}) = 1.40 \text{ \AA}$; $r(\text{F}^-) = 1.33 \text{ \AA}$)¹². To examine the first two possibilities more decisively it would be necessary to control Na content and the Cl/O ratio independently by using, for example, NaCl as a sodium source and KClO_4 as an oxygen source. Moreover, to clarify the composition and structure definitively, detailed structural analyses by X-ray and neutron diffraction are required.

Thus, we find that superconductivity in the CuO_2 planes can arise in the absence of apical oxygen atoms. The contribution to the electronic state of the CuO_2 planes from the apical Cl^- ions is sure to be different from that of O^{2-} ions. Photoemission spectroscopy for $\text{Sr}_2\text{CuO}_2\text{Cl}_2$ indicates that the Cl 3p level is located well below the Fermi level (~ 6 eV), and thus its contribution to the band near the Fermi level would be quite small¹⁵. A

similar situation may be realized in the case of the apical F^- ions in $Sr_2CuO_2F_{2+\delta}$. Our observation of superconductivity in $(Ca, Na)_2CuO_2Cl_2$, together with that in $Sr_2CuO_2F_{2+\delta}$, should provide a critical constraint on the structural conditions necessary for high- T_c superconductivity. □

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Fast photoconduction in the highly ordered columnar phase of a discotic liquid crystal

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THE search for organic materials suitable for electronic applications dates back to the early 1950s. But the only organic systems known so far to show electronic charge-carrier mobilities comparable to the amorphous inorganic semiconductors that are the mainstay of the microelectronics industry are zone-refined organic single crystals^{1–4}. Single crystals are difficult and costly to process, however, and are not suitable for device applications. Here we show that a highly ordered columnar (stacked) phase of disk-like organic molecules can exhibit high mobilities for photoinduced charge carriers, of the order of $0.1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ —higher than for any organic material other than single-crystal phases. Specifically, we study the helical columnar phase of 2,3,6,7,10,11-hexahexylthiotriphenylene, which can be prepared simply by cooling the isotropic liquid melt via the discotic liquid-crystal phase, in which the molecules are already stacked with a high degree of order.

Liquid crystals are one of the few classes of organic materials to have entered the field of sophisticated electronic applications, in particular as displays. This is due to the fact that they can be orientated in applied electric and magnetic fields. Recently, it was shown that the columnar phase structure of discotic liquid crystals is suitable for fast transport of photogenerated charge carriers⁵. Although their structural properties resemble the columnar packing of some polycyclic aromatics in the crystalline state, for example charge-transfer crystals, charge-carrier mobilities in the liquid crystalline systems are still at least two orders of magnitude slower than those in organic single crystals^{1–4} and compare to those of the very fastest—but still disordered—

molecularly doped^{6,7} or conjugated polymers^{8–10}. The short range of the intracolumnar order and considerable molecular fluctuations within the liquid crystalline phase are probably the limiting factors as compared to perfectly ordered single crystals.

We now find that 2,3,6,7,10,11-hexahexylthiotriphenylene (HHTT, Fig. 1) overcomes the above limitations by forming a self-organized, helical columnar (H) phase with crystalline order. It is most remarkable that the H phase is obtained, without major perturbations by grain- and domain-boundaries, just by cooling the system from the isotropic liquid melt. The H phase forms via a discotic hexagonal columnar (D_h) liquid crystalline phase. It is worth mentioning that the $D_h \rightarrow H$ transition is not accompanied by any symmetry changes of the two-dimensional hexagonal lattice. The overall phase sequence is described in the legend to Fig. 1.

The photocurrent transients shown in Fig. 2 were obtained by using a time-of-flight method described in Fig. 2 legend. The form of the photocurrent in the H phase—and also in the conventional D_h phase—can be explained by assuming gaussian transport. This involves a narrow charge-carrier package drifting across the sample at a constant velocity, broadened only by 'normal' thermal diffusion leading to a moderate broadening of the photocurrent decay when the package reaches the counter electrode. This is in contrast to a dispersive behaviour as found in the polycrystalline (K) phase (Fig. 2). Here, a charge-carrier package is heavily smeared out in time and space because of trapping events, resulting in a featureless current decay. Finally, the current shape in the isotropic (I) phase shows a crossover from gaussian to dispersive behaviour, the transport now being governed by shallow trapping events¹¹.

The hole mobilities as a function of temperature and for various phase regimes are shown in Fig. 3. Starting in the liquid melt, where nematic-like (orientational) short-range order is still preserved¹², carrier mobilities are in an intermediate range of the order of $10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. At the transition into the liquid crystalline D_h phase, a hexagonal columnar arrangement of molecules is established. This is reflected in the charge-carrier mobilities as a step-like increase of one order of magnitude at the phase

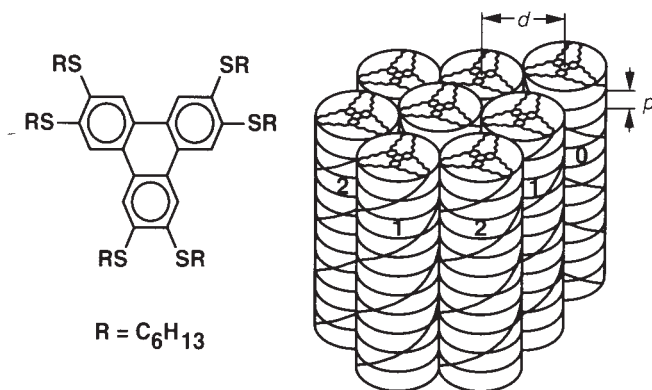


FIG. 1 Molecular structure of 2,3,6,7,10,11-hexahexylthiotriphenylene (HHTT) and the proposed structure of the helical columnar (H) phase^{13,14,18}. Column 0 is displaced by $p/2$ from columns 1 and 2, and has the opposite helicity to columns 1 and 2. The intracolumnar spacing is $p = 3.638 \text{ Å}$ and the intercolumnar spacing is $d = 21.74 \text{ Å}$. HHTT is one of the few (if not the only) known discotic mesogenic systems with both short-range intracolumnar stacking in a conventional discotic hexagonal liquid crystalline phase (D_h) and long-range intracolumnar order in the above described H phase. In addition, HHTT also has a normal polycrystalline (K) and an isotropic (I) phase; the overall phase sequence is K 62°C H 70°C D_h , 93°C I. The temperatures represent the respective phase-transition temperatures^{12–14}. For a detailed description see refs 13, 14. The HHTT molecules were synthesized according to ref. 19 and thoroughly purified by several recrystallizations.

transition. A further increase of the charge-carrier mobilities with decreasing temperature within the D_h phase is correlated with a further increase in intracolumnar correlation—which is on a short-range scale—as concluded from X-ray data^{13,14}. The mobility values in the D_h phase are comparable to those which have been observed for hole transport in the liquid crystalline D_h phase of a related material, namely 2,3,6,7,10,11-hexapentyloxytriphenylene⁵.

At the transition to the H phase, the charge-carrier mobilities again increase by almost two orders of magnitude and reach values of the order of $0.1 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$. With the exception of organic single crystals¹⁻⁴, these are the highest electronic mobility values for photoinduced charge carriers so far reported for organic systems. The mobilities in the phase are independent of both temperature and the externally applied electric field, over the accessible field range from 2×10^2 to $2 \times 10^4 \text{ V cm}^{-1}$. This is in agreement with extensive X-ray data of the phase that have shown that intracolumnar order exists on a long-range scale and that this order is not affected by temperature^{13,14}.

These mobility data in the H phase, together with the time dependence of the photocurrents (Fig. 2), suggest an efficient charge-transport mechanism with no major perturbation by disorder effects. Presumably the high mobility results from the fact that in the H phase the triphenylene units lie face-to-face with good π -orbit overlap along the columns. A comparison with the dispersive transport in the polycrystalline K phase shows that charge transport in the highly ordered H phase is the result of a very delicate balance. The phase transition from the H to the

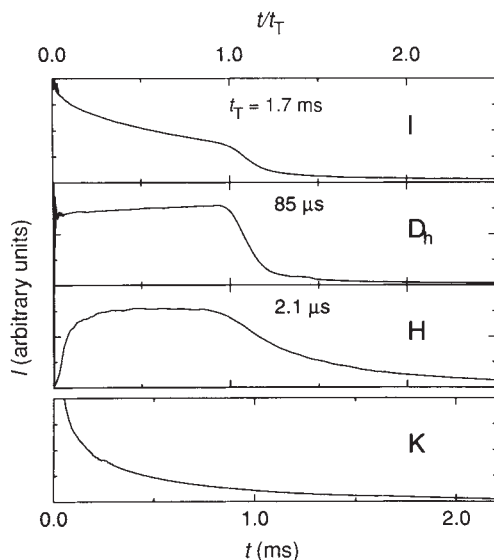


FIG. 2 Photocurrents I for holes as a function of time t measured in the different phase regions of HHTT (shown in Fig. 1) by the time-of-flight method. The stepped current decays in the I, D_h and H phases mark the respective transit time t_T of the charge-carrier package. In the K phase, a transit time cannot be determined using this straightforward procedure because of a featureless current decay. (Electric field, $2.0 \times 10^4 \text{ V cm}^{-1}$. Temperatures: I, 103°C ; D_h , 79°C ; H, 65°C ; K, 28°C .) HHTT was contained between two glass plates which were coated with semitransparent aluminium electrodes. The interaction with the flat electrodes leads to a homeotropic orientation of the molecules, that is, the columnar long-axis is perpendicular to the glass surface. The thickness of the cells was $\sim 30 \mu\text{m}$. Pulsed (10-ns) N_2 laser irradiation (337-nm wavelength) into the main absorption band of HHTT leads to the creation of electron-hole pairs in the electrically biased cell. Depending on the polarity of the external field, electrons or holes will drift across the sample cell, causing displacement currents which can be recorded in an external circuit. Details of the sample preparation and methods are given in ref. 5.

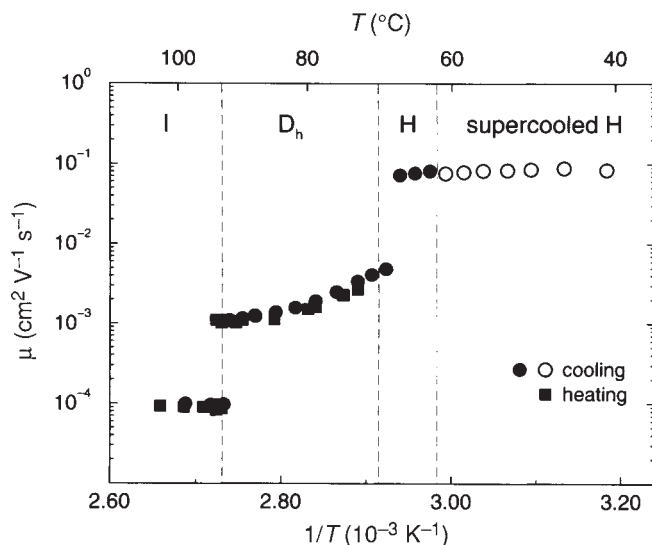


FIG. 3 Arrhenius plot of HHTT hole mobility μ versus the reciprocal of temperature T at an electric field of $2.0 \times 10^4 \text{ V cm}^{-1}$. Filled circles correspond to a cooling cycle starting in the isotropic phase, open circles were measured in the supercooled H phase; here, at 40°C the supercooled H $\rightarrow$ K phase transition took place resulting in the dispersive current decay as shown in Fig. 2 (K phase). The filled squares show measurements obtained for a heating cycle starting in the K phase; in this case, carrier mobilities cannot be obtained both for the K and H phase because of featureless dispersive photocurrents. The mobility μ is calculated using the well known relation $\mu = l/t_T E$, where l is the sample thickness, E the electric field strength and t_T the transit time (see also Fig. 2).

K phase—which can be supercooled by 20°C —is accompanied by somewhat reduced molecular dynamics and by a minute rearrangement of the respective molecular positions. Yet these small changes in structure inhibit efficient charge transport, and result in severe charge-carrier trapping. We believe that the columnar ‘transport channels’ that traverse the sample in the H phase (and also in the D_h phase) are somehow disarranged in the crystallization process, possibly by the formation of grain boundaries. The latter suggestion is supported by charge-carrier mobility measurements with increasing temperature, starting in the polycrystalline K phase. Here the photocurrent transients in the H phase still remain dispersive—like in the K phase. Intracolumnar order on a macroscopic scale can obviously not be achieved when entering the H phase via the K phase. Further heating into the D_h phase then gives essentially the same charge-carrier mobilities as found in the cooling cycle.

Our results demonstrate that disk-like mesogenic molecules can change from the liquid phase via a clearly characterized liquid crystalline phase into a highly ordered H phase, while preserving their structural symmetry and enhancing their electronic properties. As in the field of organic metals¹⁵, these results will hopefully stimulate physicists and organic chemists to further understand the interesting and potentially useful electronic properties of this new class of organic photoconductors. Their enhanced electronic properties may lead to higher speeds in the more conventional application of electrophotography, but also to novel applications, such as active layers in light-emitting diodes¹⁶ or all-organic transistors¹⁷. These examples go far beyond the present-day applications of organic solids which are mainly based on their dielectric and orientational properties. The need to maintain the temperature of the monomeric model-compound in the range of the H phase or mesophase order can probably be overcome by freezing-in the H phase or mesophase order in the glassy state of an oligomeric or polymeric system, result-

ing in a mechanically stable material for room-temperature operation. □

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Minimal effect of iron fertilization on sea-surface carbon dioxide concentrations

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It has long been hypothesized that iron concentrations limit phytoplankton productivity in some parts of the ocean^{1–3}. As a result, iron may have played a role in modulating atmospheric CO₂ levels between glacial and interglacial times⁴, and it has been proposed⁵ that large-scale deposition of iron in the ocean might be an effective way to combat the rise of anthropogenic CO₂ in the atmosphere. As part of an experiment in the equatorial Pacific Ocean⁶, we observed the effect on dissolved CO₂ of enriching a small (8 × 8 km) patch of water with iron. We saw significant depression of surface fugacities of CO₂ within 48 hours of the iron release, which did not change systematically after that time. But the effect was only a small fraction (~10%) of the CO₂ drawdown that would have occurred had the enrichment resulted in the complete utilization of all the available nitrate and phosphate. Thus artificial fertilization of this ocean region did not cause a very large change in the surface CO₂ concentration, in contrast to the effect observed in incubation experiments³, where addition of similar concentrations of iron usually results in complete depletion of nutrients.

Although our experiment does not necessarily mimic all circumstances under which iron deposition might occur naturally, our results do not support the idea that iron fertilization would significantly affect atmospheric CO₂ concentrations.

The experiment was performed in the equatorial Pacific approximately 500 km south of the Galapagos Islands. The equatorial Pacific is one of the 'high nutrient, low chlorophyll' regions of the world's oceans (others are the subarctic Pacific and the Southern Ocean⁴). In these areas biological activity does not completely remove nitrate and phosphate from surface water. New production in these waters is therefore less than it would be if all the nutrients were consumed, while the CO₂ content of the waters is substantially higher. This leads to higher levels of CO₂ in the undisturbed atmosphere than would otherwise be the case⁷. Martin^{3,4} hypothesized that the root cause of this was limitation by iron, but others have suggested that tight control of phytoplankton by grazing organisms⁸ or, in subpolar regions, lack of light or deep mixing^{9,10} could be more important.

In order to measure the natural variability against which any effect of the enrichment would have to be distinguished, a chemical and hydrographic survey of the 80 × 80 km region surrounding the release point was conducted before the release. The surface water was found to be nearly uniform over the region. Table 1 shows means and standard deviations of temperature, salinity, fugacity of carbon dioxide (f_{CO_2}), total carbon dioxide (ΣCO_2) and titration alkalinity (TA) from stations at the corners and centre of the survey area; values for the last two variables normalized to constant salinity are also shown. *In situ* f_{CO_2} (that is, the value at the surface water temperature) was 440–460 μatm , the higher-than-atmospheric value indicating that the region was a strong source of carbon dioxide to the atmosphere, as is normal for the eastern equatorial Pacific.

The experiment was then initiated by releasing ~7,800 mol of iron mixed with 0.35 mol of sulphur hexafluoride (SF₆), a generally conservative and inert tracer which may be continuously detected in seawater at concentrations <10⁻¹⁶ M (refs 11, 12). Details of the release procedure are given by Martin *et al.*⁶. The purpose of the tracer, apart from tracking the patch, was to act as a surrogate for 'released iron'—the approximate concentration of iron that would have been found in a given sample had it been conservative. Because SF₆ is a volatile compound, some escape to the atmosphere will have occurred. Concentrations can, however, be corrected for this effect by calculating the rate of gas exchange from the measured wind speeds^{13,14}. Given that chemical properties in the release area were initially nearly uniform, we hypothesized that any effect of the iron release on

TABLE 1 Properties of surface layer during pre-release survey

Property	Mean	Standard deviation	No. of observations
Temperature (°C)	22.57	0.13	91
Salinity	35.36	0.03	91
ΣCO_2^* (μM)	2064.4	2.9	33
$\text{N}\Sigma\text{CO}_2^{*\dagger}$ (μM)	2043.3	2.3	30
$\text{TA}^\ddagger$ (μM)	2328.2	2.9	31
$\text{NTA}^\ddagger$ (μM)	2304.5	2.0	30
f_{CO_2} at 20 °C§ (μatm)	405.9	6.4	91

* Method described by Johnson *et al.*¹⁸. The precision of the method is 1.4 μM . ΣCO_2 is defined as the sum of the concentrations of all forms of inorganic carbon.

† $\text{N}\Sigma\text{CO}_2$ and NTA—values of ΣCO_2 and TA normalized to a salinity of 35.00.

‡ Method described by Millero *et al.*¹⁹. The precision of the method is 2 μM . TA is defined as the number of equivalents of strong acid required to titrate 1 kg of seawater to the bicarbonate end point.

§ Method was the "discrete system" of Wanninkhof and Thoning²⁰, precision 1.5 μatm . The mole fraction of atmospheric CO₂ was 357 parts per million by volume (p.p.m.v.) at the time of the experiment (C.D. Keeling, personal column).

the ecosystem would manifest itself as an emergence of a spatial pattern in these properties, correlating with SF_6 concentrations, in the days following the release¹⁵.

The patch of enriched water was sampled over a period of 10 days, using the surface underway SF_6 mapping technique described by Upstill-Goddard *et al.*¹² to locate and guide the sampling. Surveys of the patch 3 days after release are given in ref. 6. During the first ~3 days the patch remained on the surface, but during the fourth day most of the enriched water was subducted beneath an intrusion of lower salinity water moving across the area. After the subduction event, the subsurface patch was successfully tracked by discrete water-sampling casts analysed for SF_6 , although not enough spatial coverage could be obtained by this method to make detailed maps of the distribution.

In Fig. 1, the variation of f_{CO_2} (measured at 20 °C) with SF_6 concentration is shown for four periods (days 1–2, 3–4, 5–6 and 7–9) following release. We have concentrated on f_{CO_2} here as this variable shows the best signal-to-noise ratio of the carbon parameters with respect to biological uptake or release of CO_2 , and we have the most data for it. The discrete SF_6 analyses used the method described by Law *et al.*¹⁶. A downward shift in the f_{CO_2} of water in the patch was apparent from day 2 onwards, (this could be at any time between 24 and 48 hours after a given sample was fertilized, as the spreading process took 24 hours to complete). From day 3 onwards, however, all the decrease in f_{CO_2} appeared to occur in the concentration interval $(0.5) \times 10^{-15} \text{ M SF}_6$. Higher SF_6 concentrations, corresponding to released Fe $> \sim 0.2 \text{ nM}$, did not result in any further f_{CO_2} decrease. This implies that concentrations $\sim 0.2 \text{ nM}$ were sufficient to supply iron as fast as the ecosystem could use it to increase net production.

In Table 2 the means of the f_{CO_2} determinations 'in patch' and 'out of patch' are shown. 'In patch' is again defined as $\text{SF}_6 > 5 \times 10^{-15} \text{ M}$, corresponding to 'released iron' $> 0.11 \text{ nM}$

TABLE 2 Values of f_{CO_2} for surface water after iron release

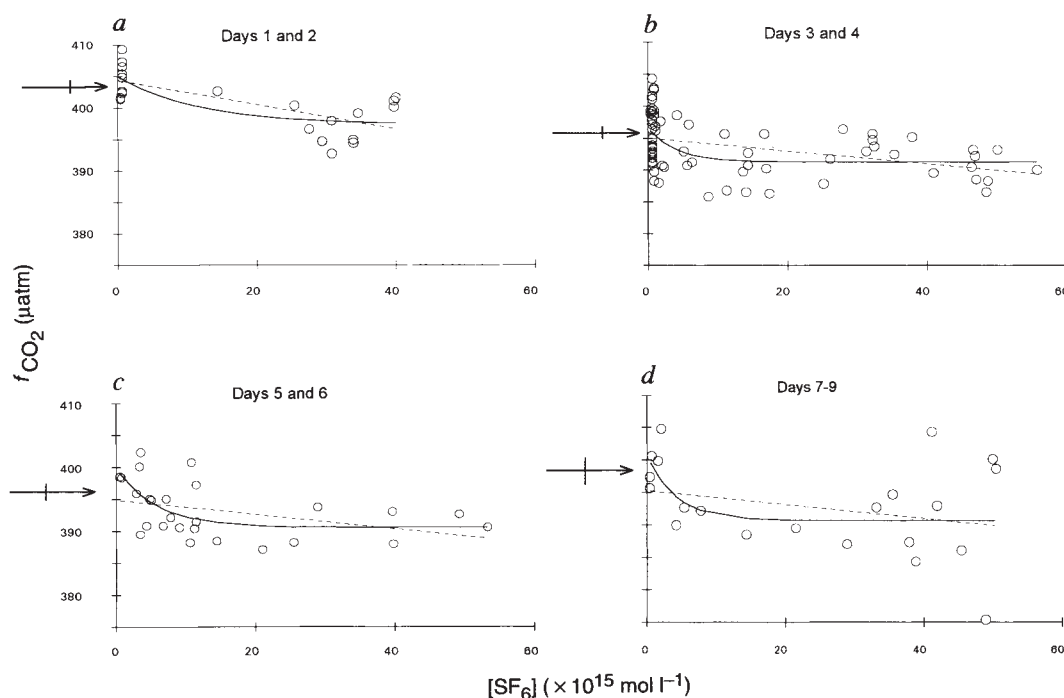
Days after release	'In patch' mean	'Out-of-patch' mean	'In'-'out' mean	P for 'in' vs 'out' samples*	'In'-'pre release'
1 and 2	398.0 (<i>n</i> = 12)	404.6 (<i>n</i> = 10)	-6.6	0.0022%	-7.9
3 and 4	391.5 (<i>n</i> = 33)	395.7 (<i>n</i> = 36)	-4.2	0.0019%	-14.4
5 and 6	391.6 (<i>n</i> = 17)	396.1 (<i>n</i> = 9)	-4.5	1.4%	-14.3
7, 8 and 9	391.1 (<i>n</i> = 17)	398.0 (<i>n</i> = 6)	-6.9	1.9%	-14.8

All values of CO_2 fugacity, f_{CO_2} (20 °C), are in μatm .

* Two-sample, two-tailed, heteroscedastic *t*-test.

immediately after release; due to the exchange to the atmosphere of SF_6 , this concentration was equivalent to $\sim 0.18 \text{ nM}$ by the time of subduction. A 2-sample *t*-test (Table 2, column 5) shows that for each period the difference in means between the 'in patch' and 'out of patch' data is highly significant. Also shown in the table are the CO_2 shifts relative to the pre-release values. From about day 3 onwards, f_{CO_2} concentrations even outside the patch decreased relative to the initial survey. The decrease occurred during the period that lower-salinity water was advecting into the study region, and may be associated with this change. There was very little mixing of the low-salinity water with the tracer-labelled patch, but the 'outside patch' values may have been affected somewhat by it. To minimize this effect, in Fig. 1 and Table 2 we used only those samples having temperature and salinity characteristics of the original patch. Another potential source of error is the lower spatial sampling density after the subduction event, which made it more difficult to

FIG. 1 Fugacity of CO_2 (f_{CO_2} , measured at 20 °C) versus SF_6 concentrations, in and around the iron-enriched patch at four periods after the release. The small pre-release background of SF_6 ($5.5 \times 10^{-16} \text{ M}$ in the surface layer) has not been subtracted. Arrows with bars show the mean and error on the mean of the f_{CO_2} values outside the patch (defined as $[\text{SF}_6] < 5 \times 10^{-15} \text{ M}$). Dotted lines are linear regressions ($f_{\text{CO}_2} = A + B[\text{SF}_6]$), solid lines are fits of the form $f_{\text{CO}_2} = a + b[1 - \exp\{c[\text{SF}_6]\}]$, where *A*, *B*, *a*, *b* and *c* are fitted by least-squares procedures. The second model would be more appropriate if f_{CO_2} approaches asymptotically a constant value with increasing iron and SF_6 concentrations. Details of the fits (with SF_6 in units of 10^{-15} M) are: *A* = 404.38, *B* = -0.193, (*r* = 0.73, *n* = 21); *a* = 404.97, *b* = -7.674, *c* = -0.0843 (*r* = 0.77, *n* = 21). Days 3 and 4, top right: *A* = 395.04, *B* = -0.101, (*r* = 0.39, *n* = 68); *a* = 396.79, *b* = -5.505, *c* = 0.255, (*r* = 0.49, *n* = 68). Days 5 and 6, bottom left: *A* = 394.86,



B = -0.114, (*r* = 0.39, *n* = 25); *a* = 399.80, *b* = -9.291, *c* = 0.169, (*r* = 0.58, *n* = 25). Days 7 to 9, bottom right: *A* = 395.31, *B* = -0.108, (*r* = 0.29, *n* = 22); *a* = 400.75, *b* = -10.10, *c* = -0.241, (*r* = 0.47, *n* = 22).

adequately sample around the patch and may introduce further error into the 'outside patch' estimates.

Changes in the other carbonate system parameters are consistent with the f_{CO_2} shifts observed. Normalised ΣCO_2 in the patch fell by $\sim 3 \mu M$ on day 1–2, rising to $6–7 \mu M$ on day 5–6, relative to the pre-release condition. There was no significant change in TA after normalization to constant salinity. Calculations of the carbonate equilibria using the values for the equilibrium constants of the seawater carbonate system proposed by Goyet and Poisson¹⁷ suggest that these values are self-consistent. The smallness of the changes is consistent with the lack of any signal in the major nutrients reported by Martin *et al.*⁶

The change in local conditions introduces some ambiguity into the interpretation of the observations; it is possible that the decrease in f_{CO_2} inside the patch relative to the initial condition was only partially due to the effects of the iron fertilization. The remainder may have been due to some other factor (perhaps associated with subduction) that would have resulted in a decrease in f_{CO_2} without our intervention. It is for this reason that in Table 2 we have calculated the shift of the mean 'in patch' values relative to both the initial survey and the mean of the 'out of patch' measurements made over the same periods. In either case a measurable shift is observed, but it is much more pronounced if the initial survey is taken as the datum rather than if the concurrent measurements outside the patch are used. In either case, the shift is apparent on day 2. In the first case it increases up to day 4 and thereafter remains constant, whereas in the second it changes little after day 2.

Whichever of these approaches is correct, the change in f_{CO_2} is very small, much less than we would have expected if the iron fertilization had resulted in a significant increase in the efficiency of nutrient uptake. The waters into which the release was made had nitrate levels averaging $10.8 \mu M$ (ref. 6). The experiment resulted in 'released iron' concentrations of $\sim 4 nM$ (ref. 6), sufficient in 'bottle' experiments to increase net production until all this excess nutrient is incorporated into organic material. If this had happened in the patch, given an organic C/N ratio of 6.6/1, carbon concentrations would have been reduced by $\sim 70 \mu M$. This would have caused reductions of $125 \mu atm$ in f_{CO_2} , calculated assuming constant alkalinity¹⁷, sufficient to transform these waters from a strong source of atmospheric CO_2 , to a weak sink. Instead the shifts we observed were between 4 and $15 \mu atm$, where the uncertainty is due to the difficulty of interpretation discussed above.

Thus although the enrichment had an easily visible effect on local productivity and chlorophyll⁶, it resulted in only a small shift in the net carbon balance compared to that which was possible. The size of the observed shift was consistent with the change in biomass observed⁶. Primary production was elevated for 6 days following the release, but there was apparently little net export of carbon from the inorganic pool after day 3, suggesting that after a brief of disequilibrium, the ecosystem responded by recycling carbon rapidly back to the inorganic form.

We conclude that an artificial fertilization of this region of ocean by addition of inorganic iron salt does not result in the realization of more than a small fraction (3–12%) of the potential for drawdown of surface water (and thus, eventually, atmospheric) CO_2 . A 'one-off' relief of iron limitation was not in this case sufficient to allow full utilization of nitrate and phosphate, although increasing the iron supply certainly affected the ecosystem and may be a necessary condition for efficient utilization of these nutrients.

Our observations argue against a role for iron fertilization as an effective strategy for combating global warming, if the behaviour we observed proves to be generally applicable to 'high-nutrient low-chlorophyll' areas. However, it is hazardous to generalize from the equatorial Pacific to other important regions such as the Antarctic. It is possible that the Antarctic ecosystem would respond in a qualitatively different way to iron

addition. It is also possible that when, as in this experiment, iron is added as a single pulse, the source is not sufficiently long-lasting to allow a very significant effect on the carbon balance, but that a more continuous source of iron would have had a larger effect on CO_2 . Both of these hypotheses could be tested by a modification of the experimental approach described here. In particular, it will be important to establish whether the length of time over which the iron is released significantly affects the magnitude of the fertilization observed. □

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Iron limitation of phytoplankton photosynthesis in the equatorial Pacific Ocean

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THE surface waters of the equatorial Pacific have unusually high nitrate and phosphate concentrations, but relatively low phytoplankton biomass^{1–3}. This 'high nitrate, low chlorophyll' (HNLC)⁴ phenomenon has been ascribed to 'top-down' grazing pressure by herbivores, which prevent the phytoplankton from fully utilizing the available nutrients⁵. In the late 1980s, however, Martin and co-workers proposed that iron, which is delivered to the remote open ocean in aeolian dust⁶, is the key factor limiting the standing crop of phytoplankton in HNLC areas^{7,8}. Using a sensitive fluorescence method⁹, we have followed changes in photochemical energy conversion efficiency^{9,10} of the natural phytoplankton community both before and after artificial enrichment with iron of a small area ($7.5 \times 7.5 km$) of the equatorial Pacific Ocean¹¹. Our

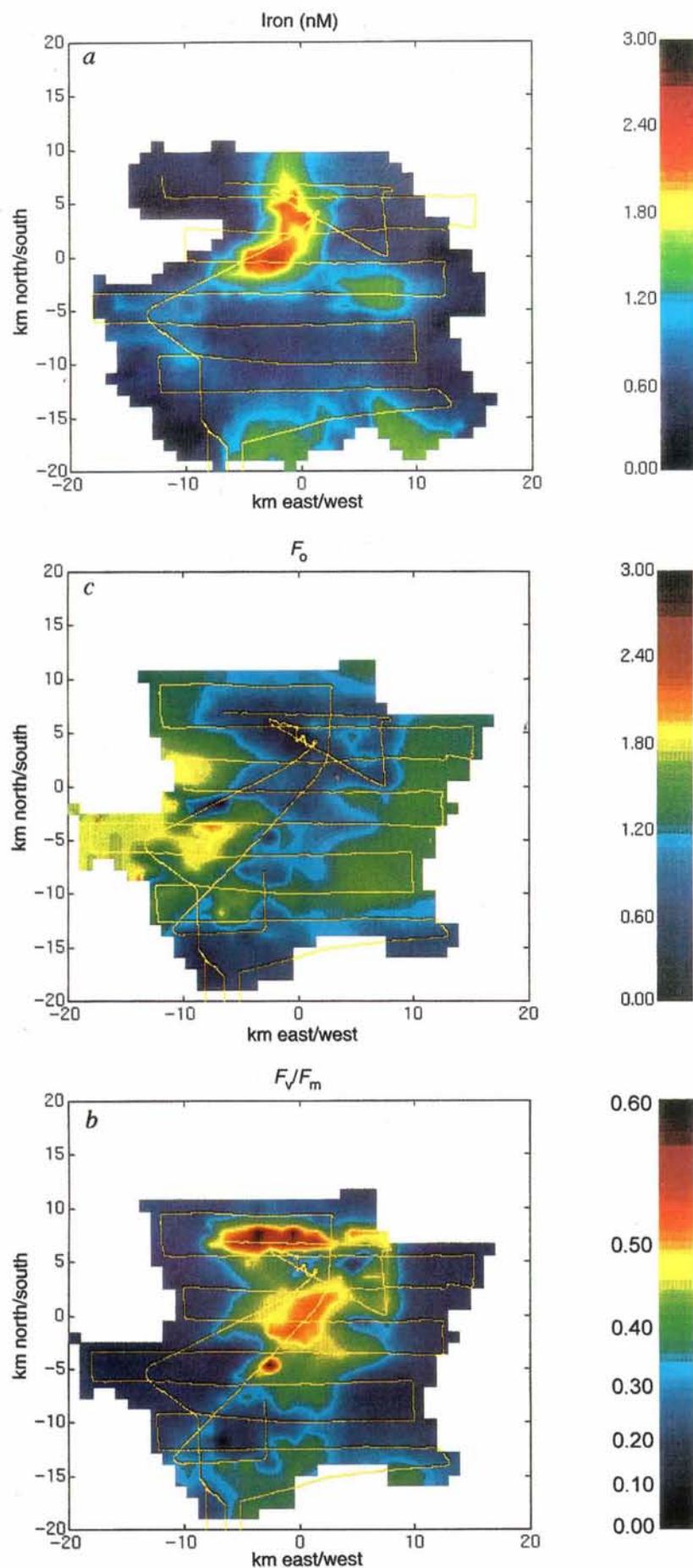


FIG. 1 a, Spatial distributions of soluble iron in the iron-enriched area at year day 300–301 (27–28 October, 1993)¹. The ship track is shown as a yellow line. The enriched area is seen as an orange-red feature in the centre of the panel. b, The corresponding variations in photochemical energy conversion efficiency (F_v/F_m) measured at 3 m below the sea surface with an onboard FRR fluorometer automatically logging data every 2 min. F_v/F_m values are spatially correlated with the iron enrichment in a. c, The corresponding spatial distribution of the minimum fluorescence yield, F_o . This parameter is markedly decreased in the iron-enriched area, implying a redistribution of absorbed excitation energy towards photochemistry in the photosynthetic apparatus of the phytoplankton.

results show that iron limits phytoplankton photosynthesis in all size classes in this region by impairing intrinsic photochemical energy conversion, thereby supporting the hypothesis of physiological ('bottom up') limitation by this element.

Following the iron enrichment (described in detail by Martin *et al.*¹¹), we continuously measured changes in photochemical energy conversion efficiency with a fast repetition rate (FRR) fluorometer as the ship steamed. The instrument operated in a flow-through mode using a non-toxic (PVC) plumbing system which sampled from 3 m below the surface. The FRR fluorometer measures the change in quantum yield of *in vivo* chlorophyll fluorescence resulting from exciting photosystem II (PSII) with a train of 32 subsaturating flashes each of $\sim 1 \mu\text{s}$ duration, each separated by $5 \mu\text{s}$. The cumulative stimulation of PSII by the fast-repetition-rate flashes induces a saturation profile of chlorophyll fluorescence at 685 nm which plateaus within $100 \mu\text{s}$ (Z.S.K. and P.G.F., manuscript in preparation). The amplitude of the saturation profile is quantitatively proportional to the quantum efficiency of PSII^{12–14}, and the saturation rate is quantitatively proportional to the functional photon absorption cross-section of the PSII reaction centre, σ_{PSII} (refs 15–17). This cross-section is a quantitative measure of the effective biophysical target size of the antenna serving PSII; the product of σ_{PSII} and incident spectral irradiance gives the average rate of photon delivery to the reaction centres. The FRR technique is extremely sensitive and the precision of the measurements is $>5\%$. To determine the effect of iron enrichment on specific phytoplankton size fractions, additional discrete samples (obtained from selected depths with trace-metal clean sampling techniques¹⁸) were size-fractionated by selective filtration and measured with the FRR fluorometer.

We previously established that, in laboratory cultures of phytoplankton, iron limitation leads to a marked decrease in the quantum efficiency of photochemistry in PSII^{19,20}. This effect, which can be conveniently and sensitively measured in real time from changes in the quantum yield of variable fluorescence^{9,14,21,22}, is independent of phytoplankton biomass loss processes, such as grazing. Thus, if iron limitation affects phytoplankton photosynthetic physiology in HNLC waters as it does in laboratory cultures, the enrichment should lead to an increase in the quantum efficiency of PSII. If, on the other hand, grazing pressure is the sole factor limiting the accumulation of phytoplankton biomass, little or no effect of iron on the quantum efficiency would be expected.

In the FRR protocol, the quantum yield of fluorescence rises from an initial, low level, F_0 , to a maximum value, F_m . The change in the quantum yield of variable fluorescence ($F_v = F_m - F_0$), normalized to the maximum fluorescence yield (F_v/F_m), is highly constrained in phytoplankton, reaching a value of ~ 0.65 under optimal growth conditions when cells are nutrient-replete^{15,16}. F_v/F_m is a quantitative measure of the quantum efficiency of PSII for an ensemble of reaction centres. We found high values of F_v/F_m in natural phytoplankton communities in high-nutrient waters of upwelling areas and continental margins¹⁶, including regions downstream of the Galapagos Islands, where currents suspend iron-rich sediments from the Galapagos platform and entrain them into nutrient-rich surface waters¹¹. In the nutrient-rich, iron-deficient equatorial Pacific, however, F_v/F_m values average ~ 0.3 , indicating a significant ($>50\%$) inactivation of PSII reaction centres and a corresponding decrease in photosynthetic energy conversion efficiency^{14,17}. Within 1 day following the addition of iron, F_v/F_m increased by $\sim 70\%$ to 0.54, and reached a peak in the centre of the enriched patch after 2 days, at a level of 0.63 (Figs 1 and 2). The increase in F_v/F_m was primarily due to decreases in the initial fluorescence (F_0) values (Fig. 1c), strongly suggesting that the iron enrichment led to an increase in the fraction of functional PSII reaction centres, and a corresponding increase in the utilization of absorbed excitation energy for photochemistry^{13,23}.

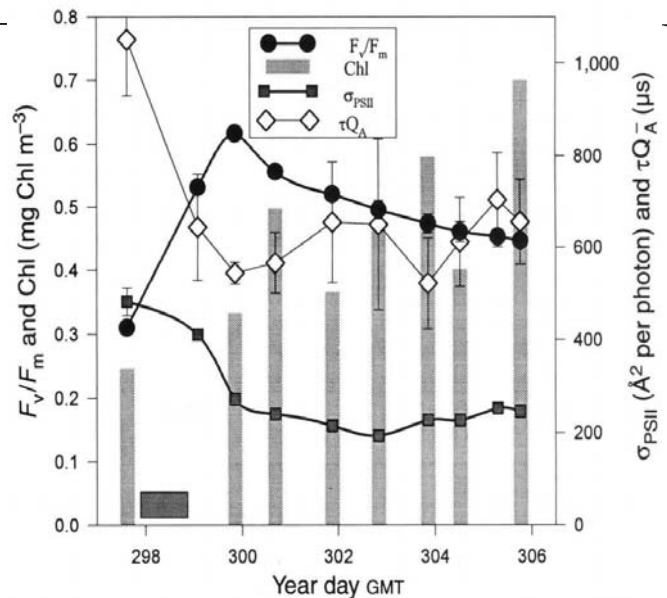


FIG. 2 The time series of changes in chlorophyll *a* (Chl), F_v/F_m , the functional absorption cross-section of PSII (σ_{PSII}), and the time constant for the oxidation of Q_A^- (τ_{QA}) following the addition of iron on year day 298 (25 October, 1993). These values are taken from the maximum of F_v/F_m in the upper 30 m of the water column. Two days following the start of the enrichment (day 300), F_v/F_m values peaked and subsequently declined with an e-folding time of ~ 6 d.

The plateau in photosynthetic energy conversion efficiency in the iron-enriched area on year day 300 (2 days after fertilization) was followed by a relaxation in F_v/F_m values (Fig. 2). The half-time for the relaxation in F_v/F_m was ~ 4 days. During the relaxation period, iron concentrations decreased from 3 nM to <0.5 nM 4 days after the iron addition. The amplitude and kinetics of iron-stimulated F_v/F_m changes were paralleled by changes in photosynthetic parameters obtained from short-term incubations with $\text{H}^{14}\text{CO}_3^-$. Based on an analysis of 34 photosynthesis-irradiance curves, the initial slopes were 19% higher inside the iron-enriched area compared with outside, while the light-saturated photosynthetic rates, normalized to chlorophyll *a*, increased $\sim 35\%$. An analysis of covariance (ANCOVA) revealed that the difference in both of these parameters was statistically significant at the $P=0.0001$ level. Moreover, based on measured optical absorption cross-sections and the values of the initial slopes of the photosynthesis-irradiance curves, the maximum quantum yield calculated for carbon fixation was 60–70% higher in the iron-enriched patch relative to the area outside the patch²⁴. Both the elevated F_v/F_m signals and enhanced photosynthetic rates were limited to the iron-enriched patch.

In addition to changes in F_v/F_m , iron affects the functional absorption cross-section of PSII^{20,25} and the rate of oxidation of the primary electron acceptor in PSII, Q_A , in model algal systems²⁰. Following the addition of iron to the waters in the equatorial Pacific, the functional absorption cross-section of PSII decreased by a factor of two, suggesting that more reaction centres became functional for a given pool of antenna pigments. Moreover, the oxidation rate of Q_A^- (which was deduced from the kinetics of the decay of variable fluorescence in the 1-ms time domain^{15,20,25}) increased by 60% within the first 2 days, suggesting an increase in intersystem electron-transfer efficiency between PSII and PSI²⁶. Similar effects of iron on PSII have been reported in phytoplankton cultures²⁰. The increase in electron-transfer rates on the acceptor side of PSII reduces the possibility of back reactions within the photosystem, leading to both an increase in the overall photosynthetic energy conversion efficiency and a reduction in the potential of photoinhibition at supraoptimal photon fluence rates.

Measurements of F_v/F_m in four size fractions spanning from $>10 \mu\text{m}$ to $<1.0 \mu\text{m}$, revealed that outside the iron-enriched

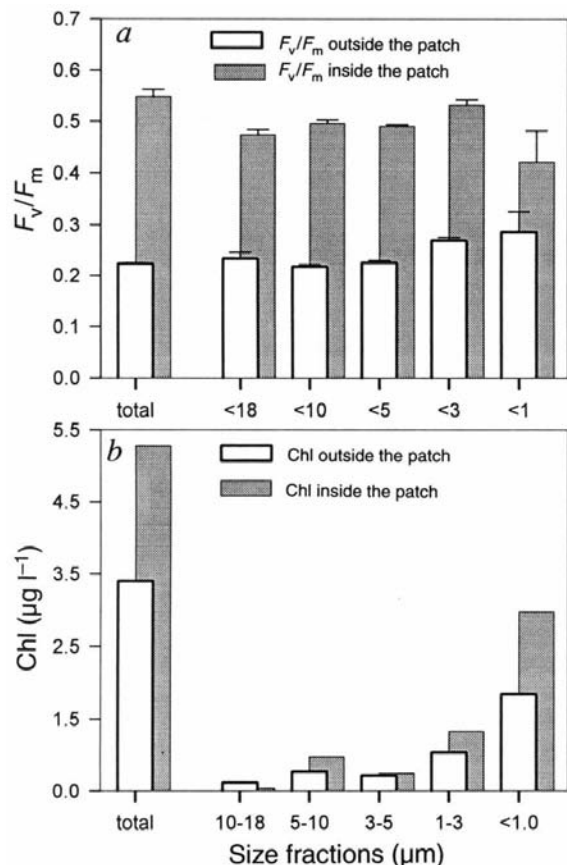


FIG. 3 Changes in F_v/F_m (a) and chlorophyll concentrations (b) in 4 size fractions inside and outside the iron-enriched patch on year day 300 (27 October, 1993), 2 days after the beginning of the iron enrichment. Note the relatively large and statistically significant increases in F_v/F_m in all size fractions in the iron-enriched waters, indicating ubiquitous physiological limitation of photosynthetic energy-conversion efficiency.

area, all size fractions of phytoplankton had low photochemical energy-conversion efficiency; average values of F_v/F_m ranged from 0.2 to 0.3 (Fig. 3a). The vast majority of phytoplankton chlorophyll biomass (>90%) was contained in small cells (<5 μm) which mainly included pico-eukaryotes, cyanobacteria and Prochlorophytes (Fig. 3b). Within 24 hours of the addition of iron, all size fractions displayed a marked increase in F_v/F_m (Fig. 3a).

Picoplankton, with their high surface-area to volume ratio, are thought to have a competitive advantage in terms of iron transport^{27,28}. Moreover, some oceanic picoplankton may have exceedingly low iron requirements per unit cell volume²⁹. The observed rapid changes in photosynthetic parameters reflect molecular alterations and/or modification of the photosynthetic apparatus and electron-transfer pathways within PSII reaction centres. That these occur in the picoplankton as well as within larger cells, implies that any refuge afforded by a high surface-to-volume ratio³⁰ is marginal in the HNLC waters of the equatorial Pacific.

The photosynthetic apparatus contains numerous loci for iron³¹. In PSII there are two iron atoms, one in the haem of cytochrome *b*-559, and a non-haem iron coordinated between the two reaction-centre proteins, D1 and D2³². In laboratory studies with iron-limited cells, fluorescence lifetimes measured in the picosecond time domain are dramatically altered by iron availability. The relative amplitudes of the two shorter lifetimes, at 155 and 530 ps, which correspond to excitation trapping and primary charge separation, respectively^{33,34} are markedly lower in iron-deficient cells compared with iron-replete cells³⁵. In contrast, a long-lived fluorescence component at 1,200 ps, which corresponds to excitation energy loss within the pigment bed³⁴,

is markedly increased. These results reveal that in iron-limited cells, there is a marked probability that excitation energy absorbed by the light-harvesting chlorophyll antenna complexes will not be trapped by the reaction centre, but rather, will be radiatively dissipated in the antenna. Western blots of photosynthetic proteins reveal large deficiencies in D1, and the two core chlorophyll antenna complexes, CP43 and CP47 relative to the light-harvesting antenna in iron-limited cells (I. R. Vassiliev, Z.S.K., D. Mauzerall, K. Wyman and P.G.F., manuscript in preparation). Addition of iron leads to an enhancement in functional PSII reaction centres, a corresponding decrease in F_0 and an increase in variable fluorescence, as more of the excitation energy is used in photosynthesis. Because of the changes in the fluorescence lifetimes, the intrinsic quantum efficiency of chlorophyll fluorescence in the phytoplankton of the equatorial Pacific is three times higher than that in non-iron-limited waters of the subtropical Pacific gyre¹⁷. Therefore, changes in fluorescence properties following iron enrichment strongly imply that iron limitation in the equatorial Pacific fundamentally affects the intrinsic molecular structure of the photosynthetic apparatus in natural phytoplankton communities.

The results presented here demonstrate that phytoplankton photosynthesis in HNLC waters of the equatorial Pacific is impaired by lack of iron, resulting in a marked reduction in the efficiency with which light is photochemically converted to stored chemical energy. The iron enrichment did not lead to a large increase in phytoplankton biomass or a correspondingly large reduction in nutrient concentrations¹¹. The latter observation might be construed to suggest that grazing maintained a low standing stock of phytoplankton. We note, however, that 2 days following the iron enrichment, photosynthetic energy conversion efficiency began to decline, suggesting that either the iron enrichment was not sufficient to sustain long-term growth, or that another trace element subsequently became physiologically limiting. Whatever the reasons for the decline, the initial increase in both the quantum efficiency of PSII and cellular chlorophyll indicate that before the iron enrichment, cells were not growing at maximum rates^{15,22}. Hence the growth of phytoplankton in the equatorial Pacific is physiologically limited by iron rather than by extrinsic factors such as grazing. □

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Seismic anisotropy and mantle flow beneath the Baikal rift zone

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SEISMIC studies have shown that continental rifts such as Lake Baikal and the Great Rift Valley of East Africa are like mid-ocean rifts in that they lie above broad regions of asthenospheric upwarp of much greater extent than the surface expression of rifting^{1–4}. The direction of mantle flow in such regions can be investigated using the seismic anisotropy created by flow-induced orientation of mantle olivine crystals^{5–8}. Seismic studies of the Mid-Atlantic Ridge have revealed upwelling mantle flow beneath the ridge and flow normal to the ridge axis on either side^{8–10}. Here we present results from an array of seismic stations across the Baikal rift zone in southern Siberia. The splitting in arrival times of SKS seismic waves indicates that the upper mantle beneath the rift zone is anisotropic, with the fast direction (which reflects the direction of mantle flow) being horizontal and normal to the rift axis. This suggests that the broad upwarp associated with this continental rift is caused by similar mantle flow to that at mid-

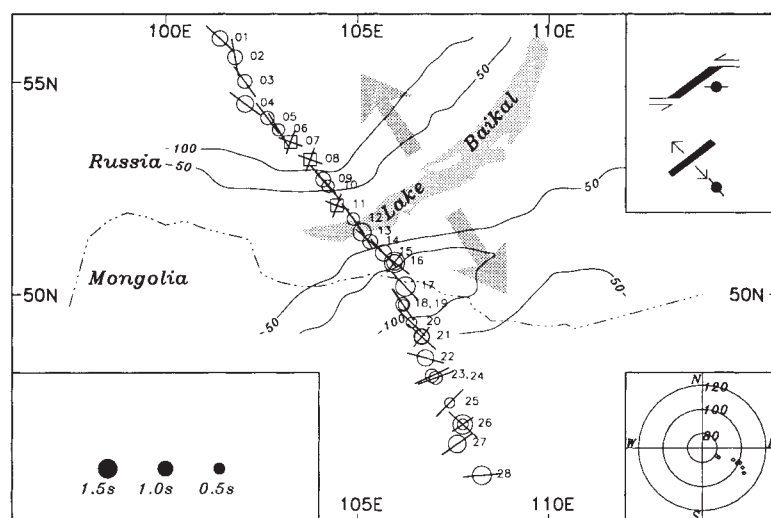
ocean rifts. This may help to elucidate the processes involved in continental rifting.

Rifting at Baikal began ~30 million years ago at an early stage of the collision between India and Eurasia¹¹. Molnar and Tapponnier¹² have suggested that the India–Asia collision generated most of the large-scale tectonics of Asia, and that the collision perhaps ripped open Lake Baikal more than 3,000 km away. In eastern Mongolia and China these authors note that deformation takes place in a shear zone of east–west, left lateral, strike-slip faults. They suggest that Baikal rifting results from a mechanism equivalent to the development of tension cracks near the ends of, and oblique to, shear zones. The diagrams in the upper-right corner of Fig. 1 show that if mantle flow beneath Lake Baikal is orthogonal to the rift^{9,13}, then the fast direction on the flanks would also be orthogonal to the rift, that is, in a northwest–southeast direction. On the other hand, as shown by the upper diagram, if rifting is a pull-apart structure within a regional shear zone¹², and this was the only motion orientating mantle olivine, the fast direction would not be orthogonal to the rift, and would most likely be east–west, because this is the direction of strain associated with the opening of the rift.

Anisotropy of the mantle is readily detected by birefringent effects on SKS phases from distant earthquakes^{14–21}. When the upward-travelling shear wave encounters anisotropic material, it may split into shear waves with two polarizations with different velocities, denoted fast and slow. The difference in arrival time between the fast and slow directions, which can amount to seconds, quantifies the degree of splitting.

In the summer of 1992 we installed 28 three-component seismic stations along the profile across the Baikal rift zone shown in Fig. 1. During the 3.5-month recording period, 9 events were found to be suitable for studying SKS phases (that is, strong events with epicentral distances >82° from the mid-point of the profile; Table 1). We have found that the effect of anisotropy can be distinguished provided the back-azimuth differs from the fast or slow directions by >10°. Although the events have a narrow range of back-azimuths (111–122°), most measurements satisfy this criterion. Figure 2 shows some seismograms with the split arrivals. We determine the two splitting parameters, fast polarization direction and splitting time, by searching for the minimum of an error function²¹. The radial and transverse components are rotated into candidate fast and slow directions, and the slow component advanced in time to be in phase with the fast component. For optimal parameters

FIG. 1 Map showing the region with the stations of the seismic array extending from the Siberian platform across the Baikal rift zone into Mongolia. Stations with well defined measurements are represented by single circles with size proportional to the splitting (see lower-left corner). The line drawn through each circle gives the fast polarization direction. Those with two inconsistent results are plotted as double circles. The shorter and longer line indicates the fast direction of the smaller and larger circle, respectively. Stations represented by squares are those on which anisotropy effects cannot be clearly observed. Contour lines are thickness of subcrustal lithosphere in kilometres, compiled from previous studies^{4,25}. Arrows are direction of extensional stress from the generalized global stress map of Zoback²². Stations 01, 11 and 28 had broadband sensors (STS-2) and the rest employed short-period sensors with central frequencies 0.5–2 Hz. During the experiment two stations (18 and 23) were relocated to nearby new sites (19 and 24). The resulting splitting parameters at old and new stations are similar. The schematic diagrams in the upper-right corner show two possibilities for the formation of the Baikal rift. Top, pull-apart basin from an east–west left lateral shear. The anisotropy symbol shows that the expected fast direction is in the shear direction east–west. Numerical simulations²⁸ indicate that almost complete alignment of olivine crystals occurs for strain >0.4. Bottom, upwelling flow in the mantle spreads out laterally from the rift. The



expected fast direction is northwest–southeast. The polar plot in the lower-right corner gives the back-azimuths and epicentral distances of the earthquakes relative to station 13 (see Table 1).

TABLE 1 Seismic events used in this study

Event no.	Date	Origin time (UT 1992)	Coordinates		Depth (km)	m_b^*	Distance [†] (°)	Back-az [†] (°)
			Lat. (°)	Long. (°)				
1	25 Jun	06:30:51.2	-28.063	-176.735	18	6.1	104.60	116.65
2	26 Jun	03:18:54.0	-33.682	-179.076	33	5.6	107.65	121.97
3	11 Jul	10:44:20.9	-22.284	-178.507	381	6.2	99.10	114.27
4	4 Aug	06:58:35.8	-21.584	-177.322	278	5.7	99.22	112.96
5	4 Aug	21:08:44.0	-12.023	-166.496	109	5.9	82.46	119.97
6	25 Aug	08:24:13.7	-20.620	-175.151	67	5.5	99.73	110.75
7	30 Aug	20:09:06.9	-17.738	-178.775	573	5.8	95.36	111.69
8	10 Sep	10:43:20.4	-22.518	-175.052	39	5.5	101.26	111.87
9	15 Sep	21:04:00.9	-14.122	167.263	196	6.1	84.61	120.51

* Body-wave magnitude. † Relative to station 13.

superposition of the two components gives a linear variation (Fig. 2). The 95% confidence intervals are determined using the method proposed by Silver and Chan²¹. The final splitting parameters (Fig. 1, Table 2) are obtained by weighted averaging according to the 95% confidence interval of each individual measurement, and show the following.

(1) Beneath the rift zone and Siberian platform, most of the fast directions are northwest-southeast, that is orthogonal to the strike of the rift, and parallel to the extensional stress direction²². (2) At the transition to the fold belt in northern Mongolia, the fast direction changes to nearly east-west, that is parallel to the faulting and fold axis. (3) The transition takes place between stations 20 and 22, over a distance of 100 km. (4) The splitting ranges from 0.3 to 1.6 s with a mean value of 0.9 s which is consistent with a layer ~100 km thick characterized by 4% anisotropy. (5) Anisotropy effects cannot be resolved at stations 07, 08 and 11. At stations 16, 21 and 26, two sets of splitting parameters fit the data equally well. Therefore, both sets are presented. One of the two fast directions is consistent with neighbouring stations.

As SKS phases have a near-vertically-incident ray path, it is not possible to determine the depth of the anisotropic layer using

values of splitting time alone. We use three approaches to constrain the depth. The first is aimed at determining how much of the anisotropy lies in the crust. It involves searching for anisotropy effects on P to S converted crustal phases²³. The observed converted S-waves in our data show no effects of anisotropy. Therefore we conclude that the layer of anisotropy is in the mantle.

The second constraint on depth concerns the sudden transition in the fast directions from nearly east-west to northwest-southeast, which occurs between stations 20 and 22. We have calculated that for an anisotropy contrast at depth, the transition at the surface occurs over a scale length given by the inner Fresnel zone at that depth. Given that the wavelength of the SKS phase is 20 km, we calculate²⁴ that the associated Fresnel zone occurs at a depth of <250 km, that is, between the asthenosphere and the surface.

The third constraint suggests that the source is unlikely to be entirely in the subcrustal lithosphere. Estimates of subcrustal lithospheric thickness based on gravity, surface waves and teleseismic P-wave delays^{4,25} range from 0 km along the rift axis to ~50 km in the regions 100 km each side of the rift axis (Fig. 1). At least in these regions, the subcrustal lithosphere is not thick

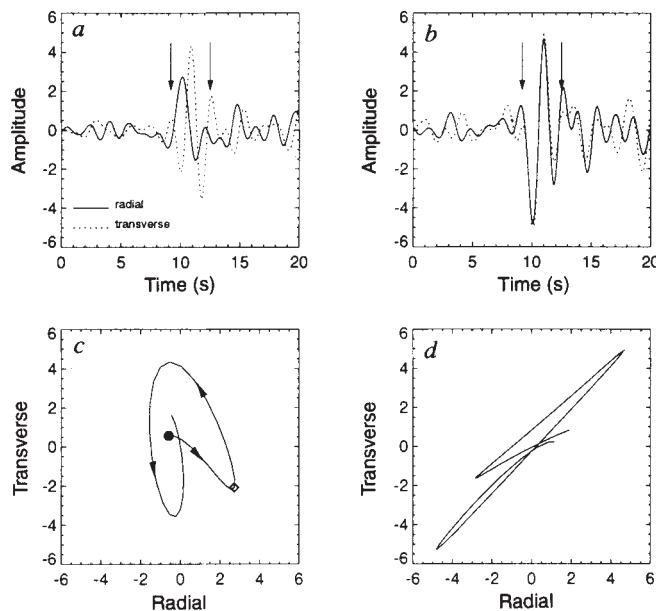


FIG. 2 Original and rotated horizontal components and particle motion patterns from event 7 for station 24. a, Original radial and transverse components. b, 'Isotropic SKS', that is, SKS arrival before entering anisotropic zone, constructed from fast and slow components, respectively. c, Particle motion pattern for the section between the two arrows in a. The arrival of the fast wave is labelled with a dot and that of the slow wave by a diamond. Arrows represent particle motion direction. d, Particle motion pattern for the section between the two arrows in b. The pattern is close to a straight line with 45° tilt angle.

TABLE 2 SKS splitting parameters and station locations

Station no.	Fast direction (°)	Splitting time (s)	Station lat. (°)	Station long. (°)	Event no.*
01	133 ± 07	1.0 ± 0.5	55.965	101.410	9
02	170 ± 13	0.9 ± 0.4	55.560	101.803	6
03	145 ± 03	0.8 ± 0.1	55.022	102.055	4, 5, 6, 7, 8
04	128 ± 04	1.2 ± 0.1	54.516	102.070	1, 2, 3, 4, 5, 6, 7, 8, 9
05	144 ± 03	0.7 ± 0.1	54.193	102.649	1, 2, 3, 6, 7, 8, 9
06	149 ± 09	0.6 ± 0.2	53.929	102.934	1, 7, 8
07a	110 ± 30	—	53.649	103.255	1, 2, 3, 7, 8
07b	020 ± 30	—	53.649	103.255	1, 2, 3, 7, 8
08a	110 ± 30	—	53.243	103.767	1, 2, 3, 7, 8
08b	020 ± 30	—	53.243	103.767	1, 2, 3, 7, 8
09	138 ± 05	0.9 ± 0.2	52.778	104.105	1, 2, 3
10	138 ± 07	0.6 ± 0.1	52.622	104.234	1, 2, 3, 4, 7, 8
11a	110 ± 30	—	52.169	104.469	7
11b	020 ± 30	—	52.169	104.469	7
12	144 ± 19	0.6 ± 0.4	51.847	104.893	2
13	148 ± 17	1.3 ± 0.3	51.526	105.121	8
14	132 ± 06	0.9 ± 0.3	51.292	105.339	6, 7, 8
15	138 ± 06	1.1 ± 0.1	51.021	105.682	1, 3, 7
16a	038 ± 04	1.0 ± 0.2	50.791	105.970	3, 7
16b	129 ± 07	1.6 ± 0.4	50.791	105.970	1, 6
17	137 ± 16	1.5 ± 0.4	50.193	106.254	6
18	145 ± 16	0.8 ± 0.1	49.747	106.188	4, 6, 7, 8
19	147 ± 24	0.3 ± 0.3	49.738	106.202	1, 2
20	134 ± 09	0.4 ± 0.2	49.288	106.412	1, 2, 3, 8
21a	039 ± 11	1.0 ± 0.4	48.931	106.682	2, 3
21b	132 ± 10	0.8 ± 0.6	48.931	106.682	1, 7
22	105 ± 06	1.1 ± 0.3	48.383	106.783	1, 2, 3, 7
23	064 ± 04	0.8 ± 0.1	47.921	106.954	1, 2, 3
24	069 ± 06	0.7 ± 0.1	47.866	107.051	4, 7, 8
25	044 ± 07	0.3 ± 0.1	47.209	107.422	3, 4, 7, 8
26a	056 ± 10	0.4 ± 0.1	46.635	107.758	4, 7
26b	132 ± 03	1.5 ± 0.2	46.635	107.758	3, 6, 8
27	055 ± 22	1.3 ± 0.3	46.115	107.619	4, 7, 8
28	085 ± 34	1.4 ± 0.6	45.262	108.260	3, 4, 6, 7, 8

* See Table 1.

enough to generate the observed 1-s splitting, unless the anisotropy is $>10\%$ which is unrealistic for subcrustal lithosphere. Therefore we conclude that, at least in the vicinity of the rift zone, the source of anisotropy is in the asthenosphere.

The fast direction for the southern part of the profile is roughly east-west. This may have been generated in a layer of mantle deformed by the collision of India and Asia. It could also be a relict from the ancient past. However, this fast direction is consistent with the dominant direction found across the Tibetan plateau²⁶. Both the Tibetan and the Mongolia plateaus have been deformed by Cenozoic deformation related to the collision. The observed fast directions in both regions may have the same origin, that is, the continental collision.

Horizontal upper-mantle flow has previously been inferred using SKS splitting at six stations positioned along the axis of the Rio Grande rift²⁷ of the western United States. The mean splitting ranges from 0.9 to 1.5 s, with the fast directions being parallel or subparallel to the rift axis. The results were interpreted as being caused by the longitudinal component of a three-dimensional small-scale convection cell associated with the Rio Grande rift. Unfortunately, comparison of this earlier study with our results cannot readily be made as the Baikal rift stations were installed exclusively along a profile across the rift and not along it.

Thus, we suggest that the observed orientation of the anisotropy around Lake Baikal is the result of uppermost-mantle flow associated with recent tectonics. The results in the far south of the study region can be explained by the shear field associated with the collision of India and Asia, whereas those from either side of the Baikal rift reflect the rift's opening. Other studies^{4,25} have shown that the asthenosphere upwarps in a broad zone extending either side of Lake Baikal, reaching the base of the crust at the lake itself. The inferred flow responsible for the upwarp is expected to remove ancient anisotropy. As at the mid-Atlantic rift, the fast direction on either side of the Baikal rift is normal to the rift axis. Measurements of vertical anisotropy along the Baikal rift axis will be required to identify whether

upwelling mantle flow also occurs directly beneath the rift as is observed on the Mid-Atlantic Ridge⁹. Lithosphere thinning caused by mantle flow would produce a zone of inherent weakness. This can explain how the stresses from the collision of India and Asia could have induced Baikal rifting¹² at such a great distance from the zone of collision. □

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Multiple processing streams in occipitotemporal visual cortex

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THE earliest stages of cortical visual processing in areas V1 and V2 of the macaque monkey contain internal subdivisions ('blobs' and 'interblobs' in layer 4B in V1; thin, thick and interstripes in V2) that are selectively interconnected and contain neurons with distinctive visual response properties^{1–10}. Here we use anatomical pathway tracing to demonstrate that higher visual areas, V4 and the ventral posterior inferotemporal cortex, each contain anatomical subdivisions that have distinct input and output projections. These findings, in conjunction with others^{11–15}, suggest that modularity and multistream processing within individual cortical areas are widespread features of neocortical organization.

We injected two retrograde tracers (three tracers in one case) into nearby sites in V4 of five hemispheres from three macaque monkeys. For three pairs of injections, strongly segregated clusters of cells labelled by each tracer were found within extrastriate visual areas V2, V3/V3A, V4v/VP and PITv (see legend to Table 1 for abbreviations). Figure 1 illustrates results from one such case (case 1 in Table 1) in which bisbenzimidazole and nuclear yellow were injected at sites 4.3 mm apart in V4 (Fig. 1a). Each injection labelled several bands of cells throughout a 14-mm swath of dorsal V2 (Fig. 1b), demonstrating that the two injections involved similar parts of the perifoveal visual field representation. The bisbenzimidazole was concentrated in the pale-staining interstripes revealed by cytochrome oxidase histochemistry, whereas the nuclear yellow was centred along a subset of the dark cytochrome-oxidase-staining stripes (red outlines in Fig. 1b). We infer that the nuclear yellow was primarily in the thin-stripe compartments (even in regions where the pattern was somewhat irregular), because it has been shown elsewhere that (1) the thick stripes do not have a substantial projection to V4 (refs 1, 3, 12, 14) and (2) anatomical segregation is maintained even within regions of irregular stripe geometry^{3,14}.

The pattern of retrograde labelling in the remainder of extrastriate cortex is shown as a three-dimensional reconstruction of occipitotemporal cortex in Fig. 1a, as a single section through the prelunate gyrus in Fig. 1c, and as a two-dimensional map of 'unfolded' cortex in Fig. 1d. Throughout occipitotemporal cortex, labelled cells were found in clusters of variable shape and size ranging from $\sim 4\text{ mm}^2$ to $>70\text{ mm}^2$.

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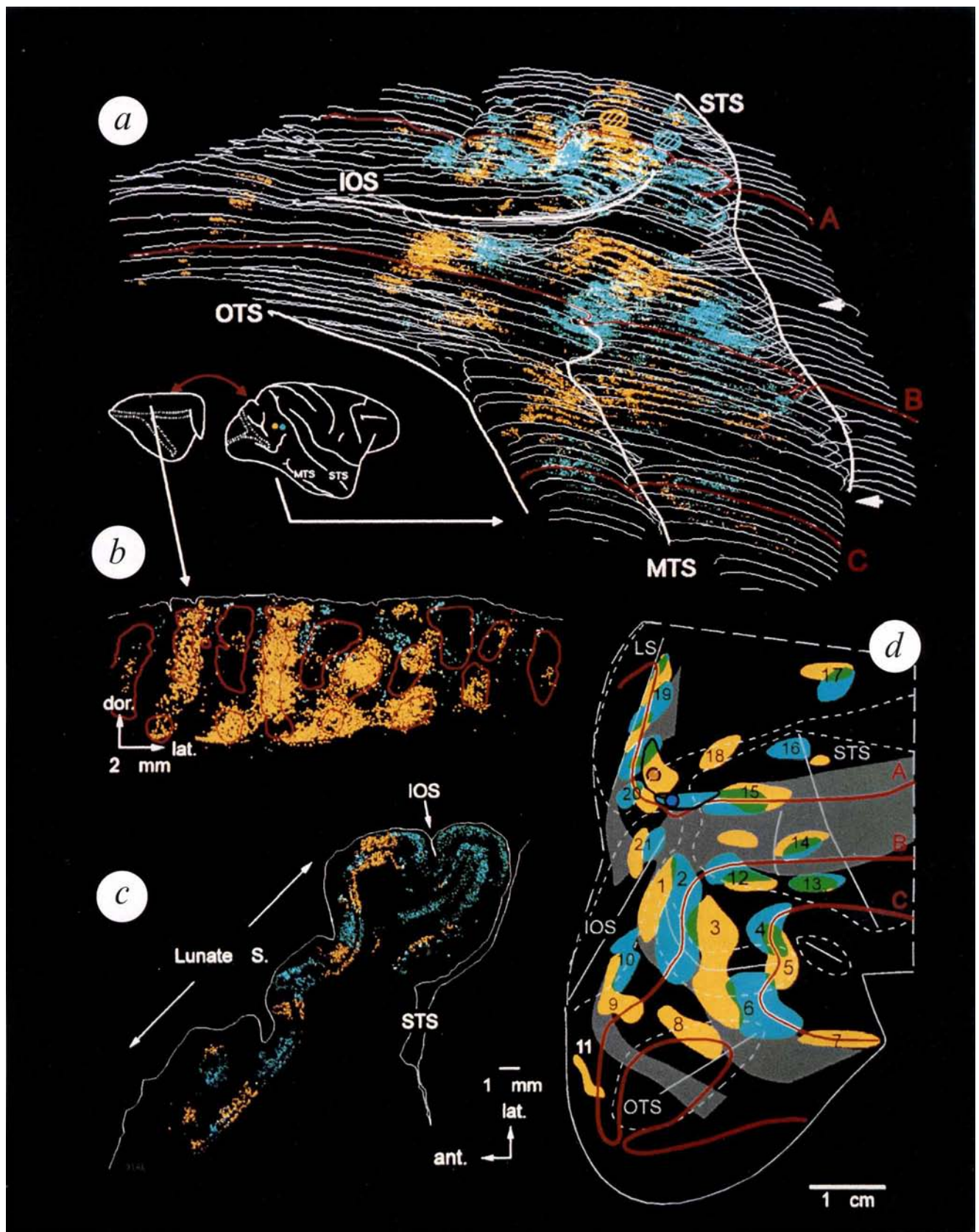
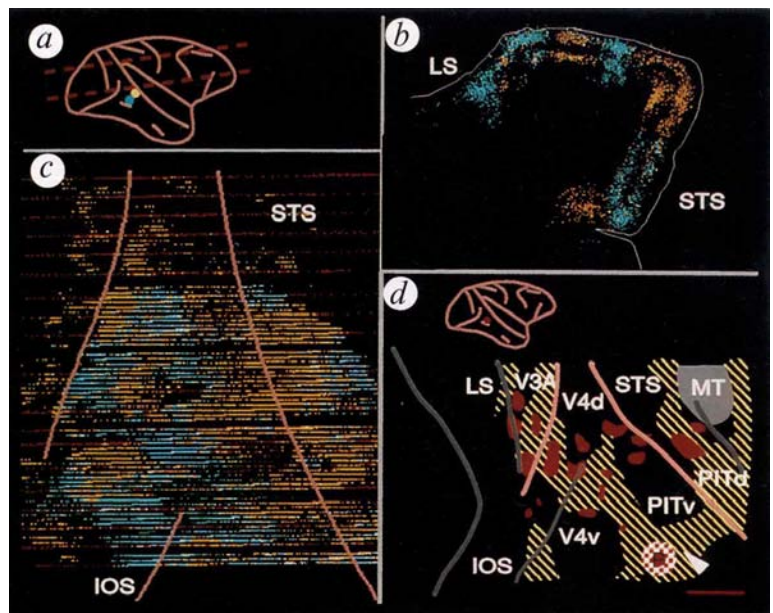


FIG. 1 Segregated, retrograde labelling of extrastriate visual areas following dual tracer injections in V4: *a*, Three-dimensional computer reconstruction of tracer labelling in occipitotemporal cortex visible from the exposed lateral surface of the brain. A Silicon Graphics IRIS workstation with custom software was used for data acquisition and visualization. Crosshatch ovals: approximate core of tracer injection sites. All dense core injection sites in this and other experiments were

restricted to cortical grey matter and were within the boundaries of the desired target area as described in ref. 16. Yellow site, 100 nl 3% nuclear yellow (Sigma) in distilled water; blue site, 100 nl 10% bisbenzimidazole (Sigma) in distilled water. Yellow and blue dots, cells labelled with the respective tracer (occasional white dots are photographic artefacts due to yellow and blue superposition). Thin white and red contours (A, B, C) outline horizontally sliced histological sections (minimum

FIG. 2 Clustering of retrograde and anterograde labelling in V4 following tracer injection in posterior inferotemporal cortex (PIT). **a**, Approximate location of tracer injection sites in PIT and range of sections shown in **c** (dashed lines). Blue dot, 250 nl injection of Fast blue (FB); white dot, 250 nl diaminidino yellow (DY) placed 5 mm more dorsal at the crown of the STS encroaching on area PITd. **b**, Computer reconstruction of labelling in three superimposed horizontal brain sections through V4 near dorsal tip of inferior occipital sulcus. **c**, Computer reconstruction of retrogradely labelled cells in 80 horizontal brain sections spanning 20 mm dorsoventrally across V4. The three-dimensional stack of sections is shown in a lateral view with the lunate, superior temporal and inferior occipital sulci outlined for reference. Major contours (red lines) are drawn at 1-mm intervals (straight horizontal gaps are due to missing sections). **d**, Anterograde labelling in a different case in which 25 μ Ci (200 nl) 3 H-proline was injected in PITv just above the posterior middle temporal sulcus (red dot on inset view). Clusters of anterogradely labelled terminals in V4 and V3A are marked by multiple red patches on an unfolded, two-dimensional cortical map. Also shown is the distribution of HRP-labelled, callosal-projecting cells (diagonal hatch) used to help identify areal boundaries^{14,30}. A, B and C: case 90DR; D, case 90CL.



In many instances, multiple patches could be assigned to individual visual areas using the pattern of interhemispheric connections (grey bands in Fig. 1d) as an independent guide to areal boundaries. For example, in inferotemporal cortex there were three large, alternating yellow and blue clusters (numbered 1–3 in Fig. 1d) which could be assigned to area PITv by virtue of their proximity to a band of callosal-projecting cells that runs along its posterior boundary¹⁴. Further anterior in the temporal lobe (to the right in Fig. 1d), area CITv also contained multiple clusters. Similarly, the anterior bank of the lunate sulcus including parts of visual areas V3, V3A and V4¹⁶ contained at least 10 distinct, alternating yellow and blue clusters (Fig. 1c, d). In contrast, labelled clusters within the superior temporal sulcus, especially anteriorly, were more intermixed, as indicated by the green zones of overlap in Fig. 1d.

To quantify the degree of segregation, we computed a segregation index designed to yield values near zero in regions of extensive intermixing and values approaching (or occasionally exceeding) unity in regions where segregation was pronounced.

spacing, 0.4 mm). White arrowheads, missing sections at block boundaries. **a** (inset), Removal of occipital cortex permits flattening of V2, exposes anterior bank of lunate sulcus. **b**, View *en face* of tracer labelling in 9 superimposed sections of dorsal V2 spanning 1.0-mm depth. Red outlines represent composite of cytochrome-oxidase-dense regions from 3 additional sections. **c**, Details of tracer labelling in horizontal section marked by red outline 'A' in **a**. Note alternating clusters along bank of lunate sulcus, including portions of V3, V3A and V4d (ref. 16). **d**, Summary map of a manually unfolded cortical map²⁸ encompassing most of the extrastriate cortex (except V2). Grey areas show concentrations of callosal projecting cells labelled with horseradish peroxidase (Sigma type VI) applied to transsected corpus callosum (some portions are occluded by overlying tracer pattern). Thin dotted lines mark cortex within sulci. Red lines mark unfolded contours of sections partially outlined in red in **a**. Yellow and blue areas indicate labelling by single tracer; green areas indicate mixing. Estimated relationships of labelled patches to visual areas: 1, 2, 3: PITv; 4, 5, 6: CITv; 7: probable TF/TH; 8: VOT; 9, 10: V4v, VP; 11: V2v; 12: PITd; 13: CITd; 14: FST; 15: V4t/MT; 16: probable MST; 17: LIP; 18: probable DP; 19: V3/V3a; 20, 21: V4. Area assignments and nomenclature are from cortical maps in refs 14, 16, 29. IOS, inferior occipital sulcus; MTS, middle temporal sulcus; OTS, occipitotemporal sulcus; STS, superior temporal sulcus (case 91AL).

Results for all V4 injections are shown in Table 1 (see legend for details of index calculation). Bold-face entries indicate regions that had segregation indices above 0.60 and that also were judged prior to quantitative analysis to have strongly segregated labelling.

In cases 1–3, strong segregation occurred in V2 and in occipitotemporal areas V3A/V3, V4v/VOT and PITv. In contrast, cases 4 and 5A resulted in consistently greater intermixing of the two tracers in V2 and in the aforementioned occipitotemporal regions. Finally, case 5B provided a valuable control condition, because the placement of injections in V4 led to separate regions of label in dorsal V2 that were the most retinotopically segregated of any case, but were predominantly within the same compartments (interstripes). This resulted in strongly intermixed labelling in occipitotemporal cortex, indicating that the degree of segregation correlated better with the compartmental distribution than with the retinotopic segregation of label in V2.

These findings suggest that some extrastriate visual areas project to V4 via at least two populations of output neurons. The projections from each population must terminate in largely segregated patches within V4, because appropriately placed injections labelled one or the other population with little intermixing. Moreover, as the degree of segregation was correlated across V2, V3A/V3 and PITv, the terminal patches derived from each area's projections must be largely coincident in V4.

To determine whether projection neurons inside V4 are also clustered in segregated domains similar to those of afferent terminals, retrograde tracer injections were made into two major targets of V4 projections, areas PITv and PITd. Figure 2a shows the location of one pair of injections placed 5 mm apart in PITv (the superior injection may also encroach on PITd). Alternating clusters of cells labelled by each tracer occupied a wide expanse of V4 within the lunate sulcus, prelunate gyrus, and superior temporal sulcus, where the labelling also included area V4t (Fig. 2b). An *en face* view of the prelunate gyrus illustrated in Fig. 2c shows the overall pattern of clustering in this case. A similar, highly segregated, yet interdigitating pattern of V4 labelling was also observed in two additional cases of dual PITv injections. In these cases, the number and size of labelled clusters ranged from three relatively large patches in one monkey to 8–10 smaller patches in another. In contrast, two more cases of paired PITv injections resulted in complex patterns of labelling in V4 which were partially segregated in some regions and partially inter-

TABLE 1 Segregation indices for extrastriate visual areas following paired tracer injections in V4

Case	Sep.	V2	V3A/V3	V4v/VOT	PITv	CITv	STS
1	4.3	0.90	0.87	0.86	0.89	0.66	0.44
2	4.5	0.92	C	0.86	0.75	0.38	0.55
3	4.0	0.93	0.86	0.61	0.84	0.37	0.52
4	4.4	0.28	0.35	0.56	0.33	0.12	0.30
5A	2.0	0.46	—	C	0.07	—	0.56
5B	4.0	0.67*	—	C	0.28	—	0.47

The segregation index for each region was calculated in several steps. First, clusters were identified on computerized reconstructions of individual histological sections (Fig. 1c for example) by initially displaying only one tracer label. Each group of cells that was clearly separated from other groups was circumscribed by a rectangular, radially aligned counting window. Groups that were not clearly separated were included in the same counting window. Next, the second tracer labelling was displayed with the first. Any cells that did not fall within the previously established counting windows were circumscribed by additional non-overlapping windows until virtually all cells were included. For each window, cells labelled by the two tracers (denoted as A_i and B_i) were then counted and a distribution index was calculated as $D_i = (A_i - B_i)/(A_i + B_i)$. This index equals zero when the cell number A_i and B_i are equal and approaches ± 1 as one or the other label predominates. Next, an overall distribution index for each visual area was calculated as $D_{\text{tot}} = (A_{\text{tot}} - B_{\text{tot}})/(A_{\text{tot}} + B_{\text{tot}})$, where A_{tot} and B_{tot} were the total number of cells labelled by each tracer within the specified visual area. D_{tot} represents the distribution index that would occur if all labelled cells in the area were uniformly mixed, and it takes into account the different numbers of cells labelled by each tracer. Departures from D_{tot} indicate distributions that were more segregated than expected. Consequently, the index of segregation for each counting window (S_i) was computed as the deviation from D_{tot} : $S_i = |D_i - D_{\text{tot}}|$. Although values of S_i can exceed 1.0 (if D_{tot} is negative), they correctly represent deviations from the uniformly mixed distribution. Final entries in the table are mean of all segregation indices (one for each counting window) calculated for each region. Bold type indicates regions with mean segregation index > 0.6 that were judged to be strongly segregated by visual inspection before quantitative analysis. C, clustered labelling, but only one tracer present. Sep, injection site separation (mm). V3A/V3, anterior bank of lunette sulcus containing area V3A and part of V3; V4v/VOT, ventral V4 plus ventral occipital temporal area and ventral posterior area if labelled; PITv, ventral division of posterior inferotemporal cortex; CITv, ventral division of central inferotemporal cortex, also including areas TF and TH when labelled; STS, superior temporal sulcus including areas MT, MST, FST and PITd. Labelling in CITd and AITd was weak but intermixed in case 1 and was too weak for classification in other cases. Case identification: 1:91AL; 2:90LR; 3:85EL; 4:91AR; 5A:90LL; 5B:90LL. * Case 5B in V2 reflects retinotopically based segregation (see text). The tracer bisbenzimidazole (Sigma) was paired with nuclear yellow (Sigma) in cases 1, 3, 4 and 5B; or with rhodamine-labelled dextrans (Molecular Probes) in cases 2 and 5A.

mixed in others. Finally, two cases of single tracer injections, one in PITv and one in PITd, resulted in V4 labelling which was partially clustered in some regions and more continuous in others.

These results show that V4 contains at least two populations of output neurons that are segregated into clusters of variable extent. Projections to PIT from each population must terminate in largely non-overlapping patches, because some paired injections labelled each population separately. Together with our previous results, this suggests that PITv itself contains anatomically heterogeneous domains.

To study the termination patterns of feedback projections from inferotemporal cortex to V4, we injected anterograde tracers into PITv. Figure 2d shows results from a ^3H -proline injection that produced a patchy distribution of terminal labelling throughout foveal V4 and adjoining regions. In two other cases, injections of wheat-germ agglutinin-horseradish peroxidase (a dual anterograde/retrograde tracer) into PITv labelled clusters of axon terminals that were often, though not always, in register

with clusters of projection neurons within V4. This indicates that in V4, as in V2^{15,17,18}, the distributions of efferent cell clusters and afferent terminal clusters are correlated but not identical.

The segregated connectivity patterns demonstrated here in occipitotemporal cortex represent an elaboration of two major processing streams which originate in the blob and interblob compartments of V1 and project to V4 via the thin- and inter-stripe compartments of V2^{2,6,19-21}. Presumably the functional characteristics of different modules in V4 and subsequently in PITv are strongly influenced by these inputs, but will be modified by additional cortical and subcortical inputs^{16,22} and by cross-connections between streams^{2,4,5,16,23-26}. The distinctions between streams may reflect a dichotomy between form and colour perception⁶, or alternatively between analyses of object boundaries versus interior surface characteristics^{21,27}. Regardless of their specific functions, multiple processing streams are characteristic of individual visual areas at four successive stages of the cortical hierarchy, presumably providing greater flexibility and dynamic control of the multiple types of analysis performed within each area. □

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Involvement of D2 dopamine receptors in the nucleus accumbens in the opiate withdrawal syndrome

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THE nucleus accumbens is prominently implicated in the reinforcing effects of abused drugs¹⁻⁴, and is an important site for mediating aversive stimulus properties of opiate withdrawal⁵. It is generally thought, however, that the role of the accumbens is negligible in the somatic signs of opiate withdrawal⁵⁻⁷. Contrary to this assumption, we now report that D2 dopaminergic receptor activity in the accumbens area potentially regulates somatic symptoms of opiate withdrawal. We find that activation of D2 receptors within the accumbens prevents somatic signs of naloxone-induced opiate withdrawal and, conversely, that blockade of accumbal D2 receptors in opiate-dependent animals elicits somatic withdrawal symptoms. These data indicate that dopamine in the accumbens not only is important in the rewarding effects of abused drugs, but also (via D2 receptors) plays a pivotal role in opiate withdrawal.

Brain nuclei prominently associated with somatic symptoms of opiate withdrawal include the locus coeruleus, central grey and dorsal thalamus⁷⁻¹². Dopamine systems may also be involved as naloxone administration to morphine-dependent animals reduces extracellular dopamine concentrations in the ventral tegmental area and nucleus accumbens^{13,14}. Furthermore, systemic treatments that prevent opiate withdrawal symptoms, for example clonidine, also prevent the decrease in accumbal dopamine levels¹³.

We tested the possibility that decreased accumbal dopamine levels contributed to opiate withdrawal by administering dopaminergic drugs and measuring withdrawal behaviours in morphine-treated rats. Systemic administration of the non-selective dopamine agonist apomorphine (300 µg–2 mg per kg; Fig. 1a), or the more selective D2 agonist propylmorphine (R-NPA) (50–500 µg kg⁻¹; Fig. 1b), significantly attenuated all somatic withdrawal symptoms measured. Such pretreatment with dopamine agonists attenuated withdrawal symptoms at the lowest doses tested, and the attenuation appeared to be dose dependent. Withdrawal-induced diarrhoea was less effectively antagonized by these dopamine agonists than the other symptoms. The ability of these dopamine agonists to reduce somatic withdrawal was reversed by pretreating rats with the dopamine receptor antagonists haloperidol or flupentixol, (0.5 mg kg⁻¹, $n=10$, $P=0.32$). In contrast to the results with the D2 agonist, neither the partial D1 receptor agonist SKF 38393 (2–5 mg kg⁻¹), nor the full D1 agonist SKF 81297 (1–10 mg kg⁻¹), reduced naloxone-precipitated withdrawal symptoms ($n=10$ each, $P>0.4$).

Peripheral administration of a non-centrally-acting dopamine antagonist, domperidone (0.5–1 mg kg⁻¹), did not block the ability of dopamine agonists to alleviate withdrawal ($n=5$, $P=0.40$), indicating a central site of action. To test the involvement of the nucleus accumbens, we microinjected R-NPA (1–10 µg) into the accumbens before naloxone-precipitated withdrawal (Fig. 1c). We found that intra-accumbal R-NPA was substantially more potent in preventing withdrawal signs than when this

drug was given peripherally (Fig. 1); note that injections were made into the shell region of the accumbens (Fig. 2).

Similar injections of R-NPA (3 µg) more dorsally into the nearby striatum were ineffective (Figs 1d and 2). Furthermore, intra-accumbal microinjections of the D1 agonist SKF 38393 not only did not alleviate any withdrawal signs but increased the severity of wet dog shakes (Fig. 1d). Finally, systemic administration of the dopamine antagonist flupentixol (0.5 mg kg⁻¹; Fig. 1d) completely blocked the ability of intra-accumbal R-NPA to alleviate withdrawal. Together, these results indicated that dopamine agonists alleviated naloxone-precipitated withdrawal by activation of D2 receptors within the accumbens/ventral striatum region.

Next, we tested whether blockade of endogenous accumbal dopamine receptors could precipitate somatic withdrawal signs. In morphine-dependent animals, but not in non-dependent subjects, systemic injections of the non-selective dopamine antagonist, flupentixol, or of the more selective D2 antagonist, eticlopride (0.5–2 mg kg⁻¹ for each), precipitated several withdrawal signs, including wet dog shakes, teeth chatter, writhing and eye twitching ($P<0.01$, $n=30$). These withdrawal symptoms were manifested within 10 min of injection of the antagonist. In contrast, when the mixtures of dopamine agonist and antagonist were administered in the previous experiment no withdrawal symptoms were seen in the 15–20 min preceding naloxone administration. These findings suggest that D2 agonists and antagonists were able to offset each others' actions on withdrawal behaviours. In addition, intra-accumbal injections of either flupentixol or eticlopride (3 µg for each) precipitated similar somatic withdrawal signs in morphine-dependent animals (Fig. 3); the intra-accumbal dose needed to produce withdrawal signs was at least 50-fold lower than when given systemically. Several somatic withdrawal signs precipitated by intra-accumbal dopamine antagonists were just as severe as when withdrawal was precipitated by systemic naloxone (Fig. 3). Other signs were less severe but were still significantly greater than when a dopamine antagonist (flupentixol, 3 µg) was injected into the striatum or when vehicle was injected into the accumbens (Fig. 3).

These data indicate that D2 receptors in the nucleus accumbens area regulate symptoms associated with morphine withdrawal. Major declines occurred in all somatic symptoms with both peripheral and intra-accumbal administration of a D2 agonist; our preliminary data indicate that systemic R-NPA also reduces withdrawal aversion measured by place conditioning (data not shown). Co-administration of D2 antagonists with the agonists prevented the suppression of somatic withdrawal signs, confirming the selectivity of D2 agonist actions. Conversely, D2 antagonists, when given alone either systemically or in the accumbens, elicited withdrawal responses in morphine-dependent animals. While intra-accumbal injections may have diffused and acted at sites other than the nucleus accumbens, similar injections dorsal to the accumbens (a likely site of diffusion from accumbens injections and equally near or closer to the ventricle as accumbens sites) were ineffective.

These results indicate marked receptor selectivity in the nucleus accumbens for dopamine-mediated modulation of opiate withdrawal, which involves D2 but not D1 receptors. There are several possible reasons for this selectivity. First, D1 and D2 receptors could be segregated on separate accumbal neurons that project to different brain areas. There is, however, controversy over such possible receptor segregation¹⁵⁻¹⁸. Second, if D1 and D2 receptors are colocalized on the same neurons, biochemical findings indicate another possibility: chronic morphine treatment is associated with increased adenylyl cyclase and protein kinase A activities in the nucleus accumbens¹⁹, and like acute morphine, D2 receptor activation decreases cyclic AMP levels in several brain areas including the accumbens, whereas D1 receptor activation has the opposite effect²⁰⁻²². Thus, it is possible that D2 receptor activity offsets biochemical changes in

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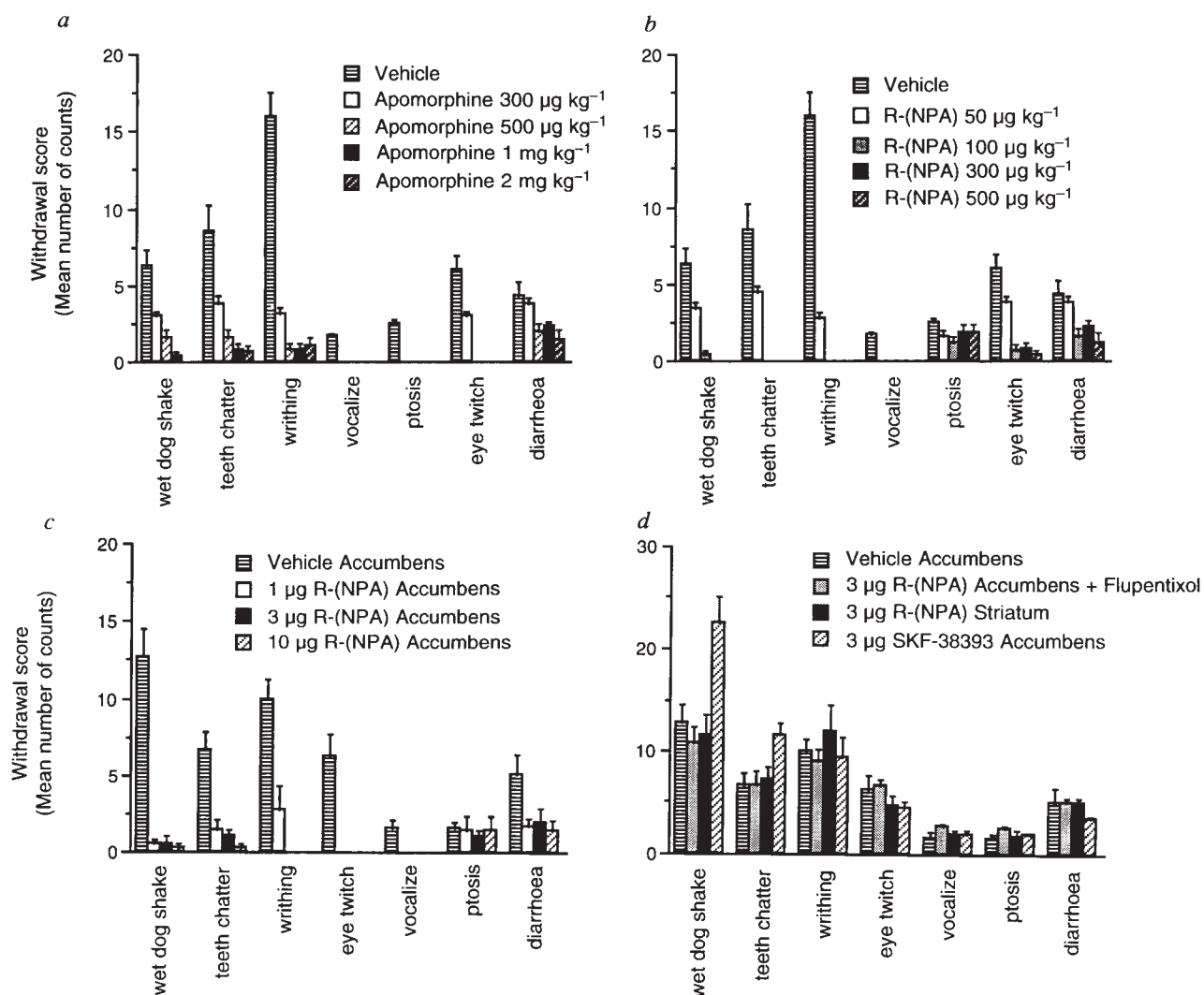


FIG. 1 Mean number of counts ($\pm$ s.e.m.) for each behaviour measured during naloxone-precipitated withdrawal. *a, b*, The effects of pretreatment (15 min before naloxone) with systemic doses of apomorphine are shown in *a*, whereas *b* shows the effect of systemic pretreatment with R-NPA. Overall $F(6, 35)$ values for apomorphine ranged from 4.58 to 72.30; $P < 0.002$ to $P < 0.0001$ for individual measurements. Doses of 0.3 to 2.0 mg kg⁻¹ were significantly different at each measure from vehicle at $P < 0.05$. For this and all other figures, scoring was performed for each measure and each treatment or dose; note that some scores were zero. Overall $F(6, 35)$ values for systemic R-NPA ranged from 6.4 to 58.56; $P < 0.001$ to $P < 0.0001$. Doses of 50–500 µg kg⁻¹ were significantly different from vehicle for measures of wet dog shakes, teeth chatter, writhing, eye twitch and vocalization, $P < 0.01$, and diarrhoea at $P < 0.05$ for doses of 100–500 µg. *c, d*, The effects of pretreatment

20 min before naloxone with intra-accumbal R-NPA are shown in *c*, and *d* shows the effects of similar pretreatment with intra-accumbal R-NPA together with systemic flupentixol (0.5 mg kg⁻¹), intra-accumbal SKF 38393 or intrastriatal R-NPA. Overall $F(6, 29)$ values for intra-accumbal R-NPA ranged from 11.25 to 23.09; $P < 0.0001$ for wet dog shakes, teeth chatter, writhing, eye twitching and vocalization. Intra-accumbal R-NPA reduced withdrawal-induced diarrhoea less than other signs, and produced a small degree of ptosis when given alone. All R-NPA doses in *c* were significantly different from vehicle; $P < 0.01$ at each of these measures except diarrhoea, $P < 0.05$. For *d*, the only significant difference from vehicle was an increase in wet dog shakes with intra-accumbal SKF 38393; $P < 0.01$. See Fig. 2 legend for details concerning intraparenchymal injections, and Fig. 3 legend for details concerning systemic injections and morphine pretreatment.

accumbens neurons evoked by chronic opiate treatment, and this normalizing effect could be responsible in part for the decrease in withdrawal behaviours observed with D2 agonist administration (but see also refs 23, 24). Further experiments are required to test these and other possibilities.

Previous studies reported that injections of an opiate antagonist into the accumbens produced only mild withdrawal symptoms⁵. Here we find that a D2 antagonist administered into the accumbens of morphine-treated animals elicited somatic withdrawal signs, some with intensities comparable to those seen after systemic naloxone. Together, these results indicate that although the accumbens may not be a major site for the initiation of opiate withdrawal symptoms, it may have an important role in regulating circuits that drive somatic and aversive responses to opiate withdrawal. Other brain structures that are responsible

for the initiation of somatic withdrawal signs may also initiate the fall in meso-accumbens dopamine.

The nucleus accumbens projects to several brain areas involved in opiate withdrawal; for example, the shell region of the accumbens has direct efferent connections to the lateral hypothalamus, thalamus, sublentiform extended amygdala, lateral preoptic area and central grey^{25,26}. It is noteworthy that the microinjections in this study were made into the accumbal shell region (Fig. 2). There may also be indirect pathways from the accumbens to other brain areas involved in opiate withdrawal. For instance, the lateral hypothalamus and amygdala have extensive projections to many brain stem autonomic centres^{27–29}.

These results call for additional research into the role of the nucleus accumbens in regulating behaviour other than locomotor activity and reward. The accumbens may have a more global

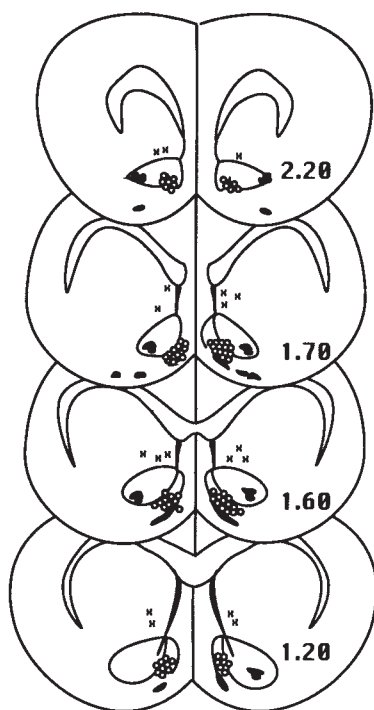


FIG. 2 Locations of all intraparenchymal injection sites for R-NPA, eticlopride or flupentixol plotted on frontal atlas sections³⁰; numbers refer to the distance in millimetres rostral to bregma for each section. Circles indicate effective sites, and crosses indicate ineffective sites. Sites for all three agents are individually plotted using the same symbols. Note that all effective sites are located in the shell region of the nucleus accumbens, while all ineffective sites are more dorsally located.

METHODS. For intraparenchymal drug tests, guide cannulae were implanted bilaterally in morphine-dependent animals 2 mm above the injection site. Cannulae were angled at 12° (dorsolateral to ventromedial) to avoid penetrating the ventricle and prevent possible diffusion of inject-

ate into the ventricle by flow upwards along the cannula. The tips of inner cannulae (30 gauge) inserted through these guide cannulae were placed in either the accumbens (AP: 1.7; ML: 3.0; DV: 7.3) or for controls in the dorsal striatum (AP: 1.7; ML: 2.0; DV: 6.0). With this approach accumbens and dorsal striatal placements were approximately equidistant from the ventricle. Following a 7-day recovery period, animals were placed under light halothane anaesthesia for the microinjections (0.8 µl volume given bilaterally, 2 min per side, $n=50$). To test the pharmacological selectivity of microinfused agonists, an antagonist was injected intraperitoneally (i.p.) immediately before halothane anaesthesia and intra-accumbal injection of the agonist. The vehicle solution was artificial cerebrospinal fluid that contained 2% ascorbic acid. Animals were allowed 20 min for recovery before naloxone (0.5 mg kg⁻¹, i.p.) was injected. Scoring for withdrawal was the same as in Fig. 1 legend. Each animal received at least two withdrawal episodes under different treatment conditions spaced 4 days apart. The order of treatments was counterbalanced. All data are from rats with injection sites histologically confirmed in the accumbens shell or dorsal striatum as indicated. Thirty-nine animals were included in the intra-accumbal injected groups, with group $n=4-5$. Nine animals had dorsal striatal injections.

role in behaviour by also regulating visceral activity associated with both appetitive and aversive stimuli. □

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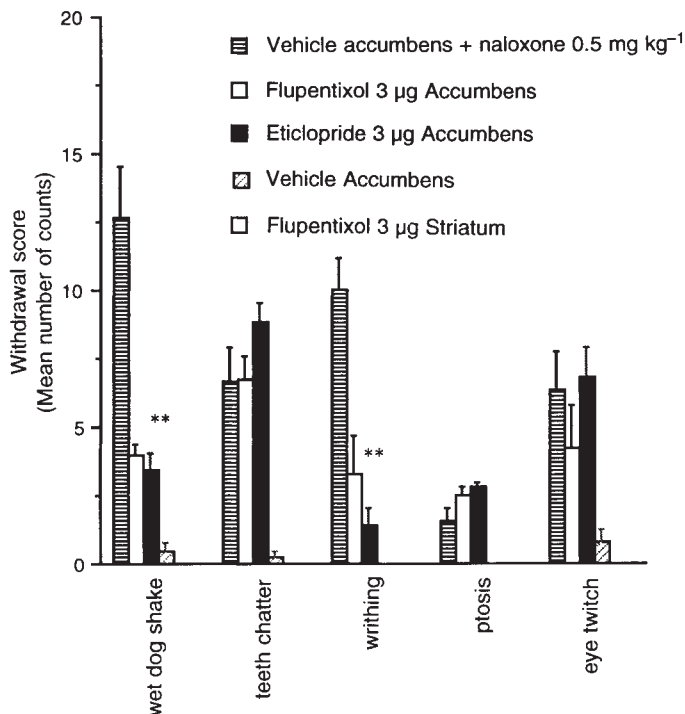


FIG. 3 Mean number of counts ($\pm$ s.e.m.) for withdrawal behaviours measured in morphine-dependent rats following intra-accumbal dopamine antagonist administration compared with systemic naloxone administration. Neither flupentixol in the striatum nor vehicle in the accumbens elicited withdrawal behaviours. A significant number of withdrawal behaviours were elicited by intra-accumbal flupentixol or eticlopride, $F(3, 21)=18.37$; $P<0.0001$. These behaviours were similar in intensity to those evoked by systemic naloxone except for wet dog shakes and writhing.

METHODS. For morphine treatment, male Sprague-Dawley rats were injected i.p. once daily at 4:00 p.m. Doses started at 10 mg kg⁻¹, and incremented by 10 mg kg⁻¹ every day until rats received 80 mg kg⁻¹ per day. After this time they were maintained at 60 mg kg⁻¹ per day for the duration of the experiment (at least 14 days). Other animals were given equivalent volumes of saline (i.p.) daily for 14 days. For systemic drug tests, 15 min before receiving a 0.5 mg kg⁻¹ i.p. dose of naloxone rats were pretreated with either vehicle, apomorphine (0.3-2 mg kg⁻¹, i.p.) or R-NPA (50-500 µg kg⁻¹, i.p.; $n=6-7$ per group). To test the selectivity of agonist effects using antagonists, a mixture of agonist plus antagonist was coadministered. The vehicle solution contained saline with 2% ascorbic acid. Instances of the following behaviours were counted for 30 min following naloxone administration: wet dog shakes, teeth chatter, writhing, eye twitching, and diarrhoea. In addition, every 10 min animals were scored for the presence or absence of vocalizing on touch, or ptosis. No instances of jumping, rhinorrhoea or lacrimation were observed during precipitation of withdrawal with this treatment paradigm. Approximately 60% of the animals were scored by a blinded observer. As there were no significant differences in the scoring between blinded and non-blinded observers, these data were pooled.

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Identification of a translocated protein segment in a voltage-dependent channel

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VOLTAGE-GATED channels undergo a conformational change in response to changes in transmembrane voltage. Here we use site-directed biotinylation to create conformation-sensitive sites on colicin Ia, a bacteriocidal protein that forms a voltage-sensitive membrane channel, which can be monitored by electrophysiological methods^{1,2}. We investigated a model of gating developed for the partly homologous colicin E1 that is based on the insertion of regions of the protein into the membrane in response to *cis*-positive voltages³⁻⁶. Site-directed cysteine mutagenesis, followed by chemical modification, was used to attach a biotin molecule covalently to a series of unique sites on colicin Ia. The modified protein was incorporated into planar lipid membranes, where the introduced biotin moiety served as a site to bind the water-soluble protein streptavidin, added to one side of the membrane or the other. Our results show that colicin gating is associated with the translocation across the membrane of a segment of the protein of at least 31 amino acids.

Biotin was introduced at positions 511, 524, 534, 537, 540, 541 and 547 in colicin Ia via seven separate single-cysteine mutations (Fig. 1; see ref. 7 for the complete sequence); the introduced biotin moiety had little effect on channel behaviour in planar lipid bilayers. In every case, the channel responded to streptavidin added to the *cis* compartment (the side to which colicin was added) in much the same way as previously reported for K544C-biotin channels¹: channels that were closed were prevented from opening, but channels that were only exposed to streptavidin when they were already open were unaffected (for example, Fig. 2a). Streptavidin had this effect even if all the soluble colicin was first perfused out of the *cis* compartment (leaving only the lipid-bound colicin), showing that it acts on membrane-bound (closed) channels¹. Thus, a region of the protein including, but not necessarily limited to, residues 511 to 547, is accessible to the *cis* solution in the closed state, but not in the open state.

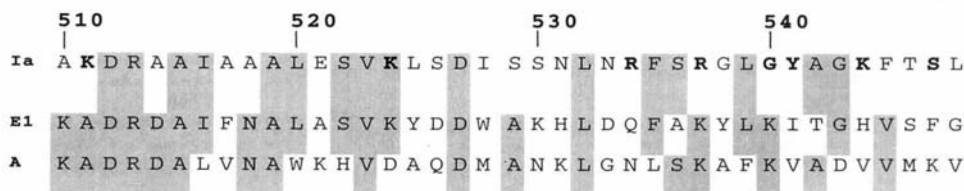
The results of exposure to *trans* streptavidin were more complex. Two of the biotinylated mutants, K544C (ref. 1) and S547C, were insensitive to *trans* streptavidin. The other six, at positions 511, 524, 534, 537, 540 and 541, were all able to bind *trans* streptavidin when the channels were open. Figure 2b shows a representative record with biotinylated mutant R534C. The membrane contained several hundred channels, which opened in response to +50 mV pulses and closed (to essentially zero current) within 1 s in response to -50 mV pulses. After exposure to *trans* streptavidin in the open state, the current no longer went to zero in response to -50 mV pulses. The amount of residual current at -50 mV increased with time, as the open channels interacted with the *trans* streptavidin. (Under these conditions, closed channels were unaffected.) This effect was not seen with unbiotinylated R534C colicin Ia, nor was it seen if free biotin was added to the *trans* compartment before streptavidin (data not shown). Thus, the effect is the result of streptavidin binding to the biotin moiety (which must be exposed to the *trans* solution) at position 534 in the open channel state. The effect of this binding on the channel, however, was not as straightforward as the effect seen with streptavidin binding from the *cis* side, which prevented channel opening (Fig. 2a); that is, the bound streptavidin did not simply act to prevent the channel from closing. This can be appreciated by considering the 'end state' of the *trans* streptavidin experiment, after long exposure times (Fig. 2b). If the channels were simply 'locked' in the open state, one would expect a symmetric d.c. current; instead, we saw an electrically noisy, highly rectifying current.

The situation was somewhat clarified at the single-channel level. After exposure to *trans* streptavidin, the channel eventually neither closed to zero conductance, as it normally does, nor remained open when the voltage was reversed from +50 to -50 mV. Instead, it appeared to flicker (at a rate too fast for us to resolve) with a mean conductance of about half the unreacted level (Fig. 2c), which would account for the rectification seen macroscopically in Fig. 2b. In fact, this behaviour was signalled by an abrupt change in the character of the open-channel noise, which may represent the streptavidin binding event (Fig. 2c, arrow). Perhaps the streptavidin molecule both interferes with normal channel gating and physically blocks the pore. Whatever the explanation, the results at the single-channel level were consistent with those at the multi-channel level, and both were dependent on the biotin at residue 534 being exposed to the *trans* solution.

The six biotinylated mutants that responded to *trans* streptavidin did so in different ways. R537C, G540C and Y541C behaved qualitatively like R534C, but the effect took longer to

FIG. 1 Partial sequence of colicin Ia in the region of the protein subjected to site-directed mutagenesis. For comparison, the aligned sequences from the corresponding regions of colicins E1 and A are included. The alignments are according to Parker et al.¹². Single-letter codes for the amino acids are used. Residues that were mutated to cysteine are shown bold. Numbers refer to the position in the amino-acid sequence of colicin Ia (ref. 7). Residues that are identical in two or more of the proteins are shaded.

METHODS. Unique cysteines were introduced by site-directed oligonucleotide mutagenesis, and the mutant colicin Ia proteins were purified and biotinylated with the sulphhydryl-specific reagent 3-(N-maleimidylpropionyl)biocytin, as described in ref. 1, and in some cases with N-(biotinoyl-N'-(iodoacetyl))ethylenediamine (both from Molecular Probes). The biotinylation procedure with the latter sulphhydryl-specific reagent was the same as that described in ref. 1, except that the buffer was 0.2 M Tris, pH 8.3, instead of 0.2 M phosphate, pH 7.0. The degree



of biotinylation was routinely assessed by two different assay systems. In an SDS-polyacrylamide gel-binding assay, the colicin band was shifted to a higher relative molecular mass when the biotinylated colicin was preincubated with streptavidin (Calbiochem) before it was loaded on the gel. Biotinylated colicin which was preincubated with streptavidin also had reduced killing activity on colicin-sensitive cells, as determined by a spot-test assay. Both of these methods routinely indicated that more than 90% of the mutant colicin was biotinylated. Furthermore, the lack of 'background' biotinylation (that is, biotinylation at sites other than the introduced cysteines) was verified by applying these assays to 'biotinylated' wild-type Ia, which contains no cysteines.

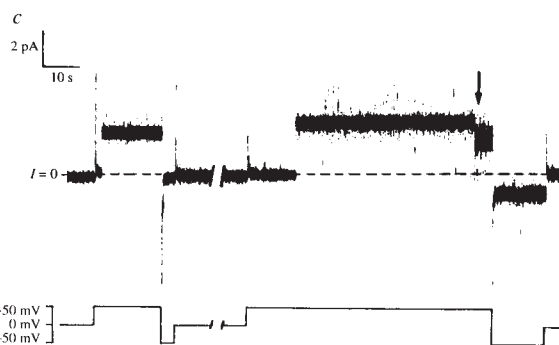
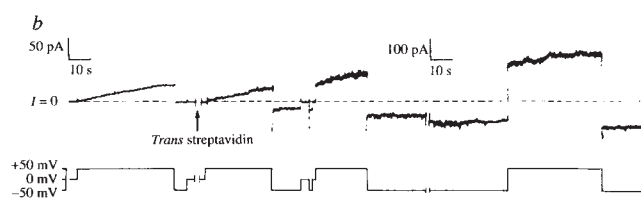
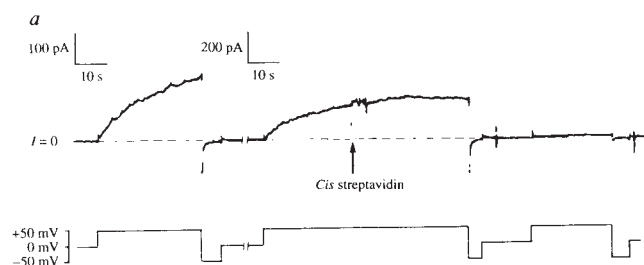


FIG. 2 The effect of streptavidin on biotinylated R534C colicin Ia (R534C biotinylated with 3-(*N*-maleimidylpropionyl)biocytin). In each panel, upper trace is membrane current, lower trace is voltage of *cis* compartment with respect to the *trans*. Membranes formed as in ref. 1. All multichannel experiments were done in 0.1 M KCl, 5 mM CaCl₂, 1 mM EDTA, 50 mM MES, pH 6.2. Transmembrane voltage was clamped and current monitored by standard techniques. Colicin (1–10 mg ml⁻¹) was diluted 1:1 with 1% octylglucoside (Calbiochem) before addition to the *cis* compartment; this yielded more channels per mg protein without affecting channel behaviour. *a*, *Cis* streptavidin. Biotinylated R534C (1.2 µg ml⁻¹) was in the *cis* compartment. At the arrow, streptavidin was stirred into the *cis* compartment to a concentration of 40 µg ml⁻¹. Channels were then closed by a brief -50 mV pulse. A subsequent +50 mV pulse turned on almost no conductance. *b*, *Trans* streptavidin. Biotinylated R534C (1 µg ml⁻¹) was in the *cis* compartment. At the first break in the record (arrow), streptavidin was added to the *trans* compartment to a concentration of 40 µg ml⁻¹. During the second break (90 s), more conductance was turned on by +50 mV pulses. Before addition of *trans* streptavidin, current rapidly went to zero when voltage was switched from + to -50 mV; afterwards, it did not do so, and there was increased noise in current records at +50 mV. *c*, *Trans* streptavidin, single-channel record. Conditions as in *a* and *b*, except [KCl]=1 M. Biotinylated R534C (15 ng ml⁻¹) was in the *cis* compartment; streptavi-

din (10 µg ml⁻¹) in *trans*. The current was digitized, recorded on videotape and filtered at 1 kHz for playback; the record shown here was made on a General Scanning chart recorder with pen response of ~100 Hz. The arrow marks the putative streptavidin binding event (see text). When voltage was now switched to -50 mV, the channel did not completely close. We never saw this sort of failure of channel closure in the absence of streptavidin (observations on 64 channel openings in six membranes); following streptavidin addition to the *trans* compartment, we observed, over time, at least 20 channels that failed to close completely.

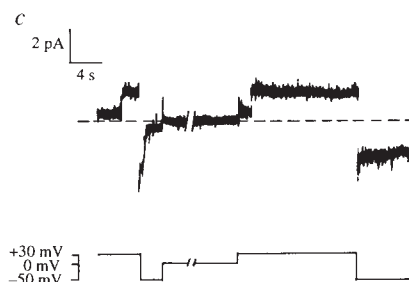
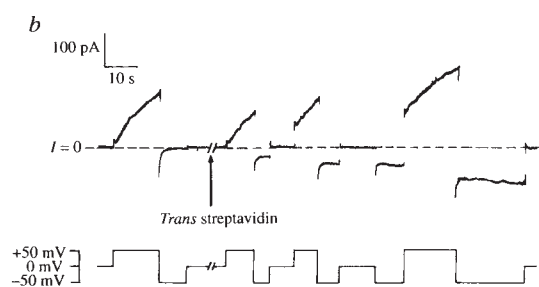
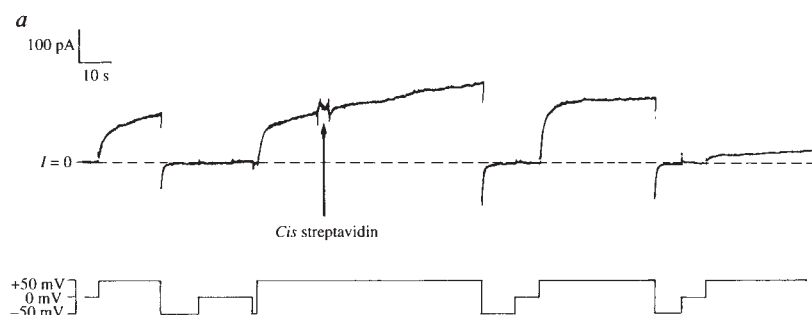


FIG. 3 The effect of streptavidin on biotinylated K524C colicin Ia (K524C biotinylated with *N*-(biotinoyl)-*N'*-(iodoacetyl)ethylenediamine). Experimental procedures and conditions as for Fig. 2, except that the buffer concentration was 20 mM. *a*, *Cis* streptavidin. Biotinylated colicin had been added to the *cis* compartment earlier in the experiment (final concentration 1 µg ml⁻¹). At the arrow, 40 µg streptavidin was stirred into the *cis* compartment. The channels were subsequently closed with a -50 mV pulse. About 85% of the channels reopened on the next +50 mV pulse. The conductance was then turned off again, followed by another +50 mV pulse, which now recruited only about 15% of the original conductance. *b*, *Trans* streptavidin. Biotinylated colicin had been added to the *cis* compartment earlier in the experiment (final concentration 0.6 µg ml⁻¹). At the (90 s) break in the record, 10 µg streptavidin was stirred into the *trans* compartment. The channels were then cycled through a series of + and -50 mV pulses. In the presence of *trans* streptavidin, the channels failed to close normally. *c*, *Trans* streptavidin, single-channel record. The recording was taken directly from a Physiograph chart record filtered at 30 Hz. The salt was 1 M KCl. Biotinylated colicin (final concentration 0.2 µg ml⁻¹) had been added to the *cis* compartment, and streptavidin (final concentration

10 µg ml⁻¹) had been added to the *trans* compartment, earlier in the experiment. The channel initially opened at +30 mV and rapidly closed at -50 mV. Later (~90 s), it subsequently opened again at +30 mV, but then failed to close at -50 mV. At some time during the latter +30-mV stimulus, streptavidin had bound to the biotinylated Cys 524. (The small current at +30 mV and -50 mV when the channel was closed resulted from the background conductance of the bilayer.)

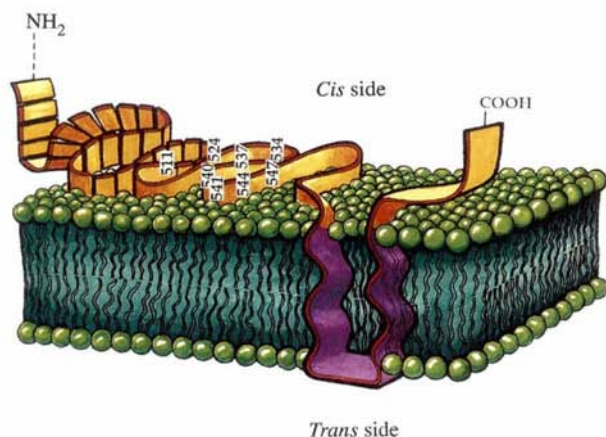
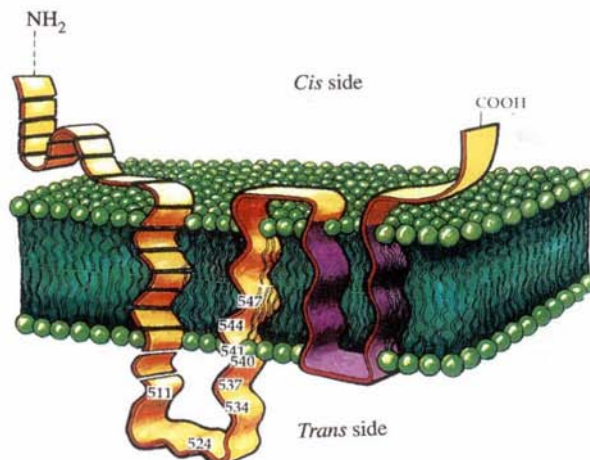
a Closed state**b** Open state

FIG. 4 Model of the colicin Ia channel in the closed (a) and open (b) configurations. The specific meanders of the polypeptide chain are hypothetical. Residues for which positional information was obtained in this work are indicated by number. The protein chain upstream of amino acid 511 is shown as a broken band to indicate the absence of data for that region. The hydrophobic region is drawn in purple. No attempt has been made to indicate how the membrane-spanning regions might be organized into an ion-conducting channel. The mobile segment investigated here is shown as crossing over from the *cis* to the *trans* solution when the channel goes from the closed to open state. In addition to its role in gating, this region of the protein is likely to be crucial for forming the channel^{6,13}. The hydrophobic segment, an α -helical

hairpin in the crystal structure of colicin A^{12,14}, is shown traversing the membrane in both the open and closed state, although its orientation is not known for either state. Data from colicin A argue against a membrane-spanning orientation^{15,16}, at least in the closed state, but data from colicin E1 have been interpreted to mean the opposite¹⁷⁻¹⁹. Colicin Ia binds to lipid bilayers nearly irreversibly, even in the closed state (data not shown), presumably through its hydrophobic region, so it seems plausible that the hydrophobic segment is largely in the membrane, and may contribute to forming the channel. There is, however, no reason to rule out the possibility that the orientation of this segment also may change as the channel gates.

develop in G540C and Y541C, perhaps because the attached biotin is less favourably exposed to the soluble *trans* streptavidin. K511C and K524C had a somewhat different behaviour. The full effect developed almost immediately on exposure of the open channel to *trans* streptavidin, and the final state of this system showed much less rectification and lacked the extra open-channel noise seen with R534C (not shown). For these two mutants at least, the major effect of *trans* streptavidin seemed to be to prevent channel closing. The complexity of the results with *trans* streptavidin undoubtedly arises from subtleties in the structure of the open channel and the function of the labelled residues in gating, but the principal finding is clear—namely, that residues 511–541 cross from the *cis* to the *trans* side of the membrane in conjunction with the opening of the colicin Ia channel.

The biotinylation reagent used above (3-(*N*-maleimidylpropionyl) biocytin) attached a biotin moiety to cysteine by a spacer arm ~16 Å long. We also biotinylated four of the mutants (K511C, K524C, R534C and Y541C) with a reagent (*N*-biotinyl)-*N'*-(iodoacetyl)ethylenediamine whose spacer arm is about 8 Å. The results, shown for K524C in Fig. 3, were fundamentally the same as those with the longer spacer arm; *cis* and *trans* streptavidin blocked the normal opening and closing, respectively, of these channels. Thus, in the most extreme interpretation, all the residues that interact with *trans* streptavidin would be 8 Å from the *trans* surface. Although the data cannot rule out such a model, it seems highly unlikely, especially as a large part of the spacer arm may be required for tight binding^{8,9}. A more conservative interpretation would allow at least some of the '*trans*' residues to be exposed to the *trans* solution, while others (perhaps those that exhibit a less robust '*trans* effect') may be sequestered in the bilayer, a few ångströms from the *trans* interface.

Figure 4 represents a plausible model incorporating the results described here. The hydrophobic segment, common to the car-

boxy-terminus of all sequenced channel-forming colicins², is shown in purple. The biotin/streptavidin experiments show that the region of colicin Ia immediately upstream from its hydrophobic segment moves into and across the membrane in conjunction with channel gating. This same region is almost certainly involved in forming the channel itself. Thus, the voltage dependence of colicin channels is due (at least in part) to the voltage-dependent insertion of structural elements of the channel into the membrane. Although these experiments do not distinguish among the many substates of the colicin channel, they do allow us to detect gross motions of the protein, which in the case of colicin Ia seem to be the basis for voltage-dependent gating.

The opening of the colicin Ia channel is associated with the translocation to the *trans* solution of a considerable length of peptide—at least 31 amino acids (the region from K511 to Y541), and perhaps more. On the downstream side of this segment, we found that residue K544 does not move completely across the membrane, which puts a limit on the maximal C-terminal extent of the segment translocated to the *trans* solution. There is no corresponding limit, at this time, on the maximal upstream extent of this segment, although we suspect that the amino terminus remains on the *cis* side. The translocation of proteins across membranes, such as occurs in organelles, is thought to be mediated by an *in situ* protein translocation complex¹⁰ although there is a theoretical basis for believing that, at least in some cases, a protein can cross a lipid bilayer by interacting directly with the lipid¹¹ as evidently occurs for certain protein toxins. Channel-forming colicins may be an example of the latter. Evidently, as colicin Ia, by utilizing the membrane potential, can translocate a substantial portion of itself in the absence of any other membrane proteins. The tolerance of the translocated region for insertions and substitutions remains to be established, but it may be feasible to make use of molecules

such as colicin Ia to inject sequences of interest across membranes. □

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Genetic mapping of a susceptibility locus for insulin-dependent diabetes mellitus on chromosome 11q

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LOCi in the major histocompatibility complex (MHC) on chromosome 6 and the insulin (*INS*) region on chromosome 11 have been implicated in susceptibility to insulin-dependent diabetes mellitus (IDDM) through candidate gene investigations^{1–5}, but they may account for less than 50% of genetic risk for the disease⁶. Genome-wide linkage studies have led to localization of more than 10 susceptibility loci for insulin-dependent diabetes in the non-obese diabetic (NOD) mouse⁷ and the BB rat⁸. Similar studies are now possible in humans through the development of dense genetic maps of highly informative microsatellite loci obtained using polymerase chain reaction analysis⁹. We have applied microsatellite markers from recent Génethon maps^{10,11}, and other highly informative markers, in a genome-wide linkage study in IDDM. Here we report evidence for the localization of a previously undetected susceptibility locus for IDDM in the region of the *FGF3* gene on chromosome 11q. Our result shows the potential of genome-wide linkage

studies to detect susceptibility loci in IDDM and other multifactorial disorders.

A panel of 314 Caucasian IDDM-affected sibling pairs from 231 families of French, American and North African origin was assembled for linkage studies. Initially, we characterized 321 markers (247 comprising an index map and 74 others from specific regions of interest) in a sub-panel of 61 affected sibpairs from French and North American families (Table 1). Markers were examined in a larger number of affected sibpairs when the significance of the linkage statistic in the 61 sibpairs was $P < 0.05$ (Table 1 legend). We decided *a priori* to create subsets of affected sibpairs for linkage analysis using the strong association of IDDM with HLA-DR3 and HLA-DR4 antigens¹ to select subsets that could represent groups with different degrees of genetic susceptibility or those influenced by different genetic factors. The linkage tests were carried out in all affected sibpairs, and in sub-categories of affected sibpairs in which both were positive for HLA-DR3 or for HLA-DR4. The HLA classification was not used in the sub-panel because it contained only a small number of affected sibpairs in these categories.

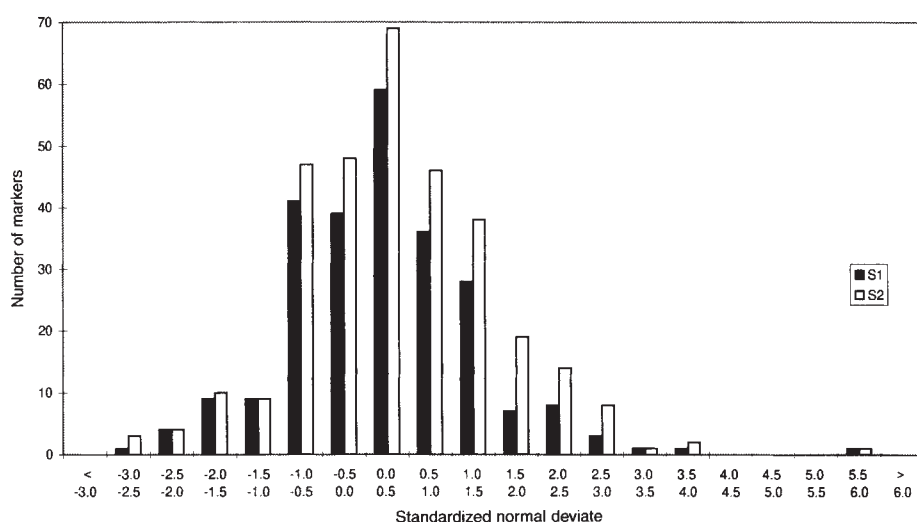
Figure 1 shows the distribution of the test statistics for index markers characterized in the 61 affected sibpairs. Nineteen (8%) of the markers gave a test statistic with $P < 0.05$ in a one-sided test for linkage based on identity-by-descent (IBD) greater than 50%, that is, higher than expected allele-sharing (see Table 1 for details; also see Supplementary Information). These markers were confined to 10 regions (with recombination distances of 20% or less between markers in the same region). In three instances, the test statistics were significant at $P < 0.001$ (for *HLA*, *D6S271* which is linked to *HLA*, and *FGF3* on chromosome 11q). With 74 additional markers, the number of markers with $P < 0.05$ increased to 40 (12%) in 15 regions. These observations can be compared for significance with the distribution of the test statistics for index loci at which IBD was $< 50\%$, that is, for which a deficiency of sharing was observed, presumably by chance. Significance at the 0.05 level was found for 10 of the index markers (4%); all were unlinked and none was significant at $P < 0.001$.

Five of the markers that showed potential linkage in the 61 affected sibpairs were near the MHC, and they were not examined further. The other 35 were characterized in additional affected sibpairs from families in which both parents could be genotyped. For 17 markers, we observed an IBD of $> 50\%$ with $P < 0.05$ when the test statistic was calculated for all 251 affected sibpairs, or in one of the HLA subsets (Table 2a). Outside the MHC, the strongest evidence for linkage was found for *FGF3* on chromosome 11q13, significant at $P < 0.005$ in all affected sibpairs, and $P < 0.0001$ for HLA-DR3-positive sibpairs. Other markers from the same region gave test statistics significant at $P < 0.0005$ in HLA-DR3-positive diabetics (Table 2a and Fig. 2). A further subdivision was made by separating HLA-DR3/DR4 sibpairs from other HLA-DR3- or HLA-DR4-positive sibpairs. Evidence of linkage was found in both HLA-DR3/DR4 and other HLA-DR3 diabetic groups, but not in HLA-DR4 diabetics (after exclusion of HLA-DR3/DR4) or other diabetics (Table 2b). Another subdivision of the data based on IBD at the *HLA* locus shows that linkage to *FGF3* occurs predominantly in affected sibpairs who share two *HLA* alleles (Table 2c), although this effect is not as strong as in the HLA-DR3-positive pairs. (The division by IBD at *HLA* is not independent of the division by HLA-DR3 and DR4 (ref. 1), and we did not use this division *a priori* to investigate linkage.) Additional support for linkage was found when data from 63 affected sibpairs in families without DNA from one or both parents were analysed (Table 2d).

IBD for affected sibpairs at the hypothesized *FGF3*-linked susceptibility locus is less than at *HLA* (58% compared with 71%). Other methods of comparison also show that *HLA* has a stronger effect than *FGF3*. For example, using the method proposed by Risch⁶, the increased risk of IDDM due to linkage to these loci in siblings of diabetics compared with the prevalence

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FIG. 1 Distribution of the test statistic, T , for linkage with markers characterized in the sub-panel of 61 affected sibpairs. Dark bars in the histogram indicate results for 247 markers from the primary map, and light bars include 72 additional markers selected because they were in areas of interest, either in conserved synteny groups from regions containing a susceptibility gene in the NOD mouse, or near anchor markers that showed a suggestion of linkage. The markers consisted principally of Génethon microsatellites^{9,10}, complemented with a small number of others (10%), mostly VNTRs (variable number of tandem repeats), chosen to extend coverage in sub-telomeric regions. The average interval spanned by adjacent markers was estimated to be 15 centimorgans (cM) in the primary map (Génethon data), and the mean heterozygosity was 0.79 in parents from the sub-panel families. After inclusion of additional markers, the average spacing was 11 cM and mean heterozygosity 0.77. The total map spans approximately 32.5 morgans, and the distance between adjacent markers was greater than 20 cM for less than 6% of intervals. Markers for which the test statistic exceeded the 0.05 critical limit (1.64) with IBD > 50% were characterized in the 251 sibpair panel with the exception of markers linked to *HLA*. Markers that gave evidence of linkage with significance $P < 0.05$ in the larger panel are shown in Table 2a. The loci and regions



that showed no evidence of linkage were (1) 1p: *D1S80*; (2) 1p: *ALPL*; (3) 1q: *SPTA1*, *D1S194*; (4) 2q: *D2S154*; (5) 5q: *D5S400*, *D5S429*; (6) 7p: *D7S506*; (7) 8q: *D8S281*; (8) 12q: *D12S85*; (9) 14p: *D14S72*; (10) 20q: *D20S171*. Other markers were in regions shown in Table 2a, but are not listed because their significance in the 251 sibpair panel was > 0.05 . Genotype data were obtained and verified as described previously¹⁰⁻¹² (with slight modifications).

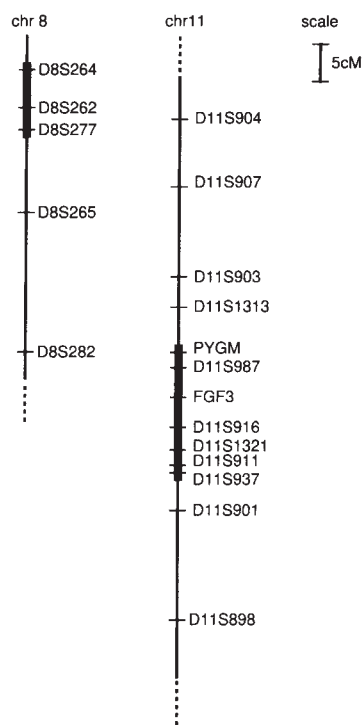


FIG. 2 Genetic maps of chromosomes 8 and 11 in regions of potential linkage to IDDM. Thick bars represent the regions spanned by markers for which the test statistics gave $P < 0.05$ in 251 affected sibpairs.

in the general population was estimated to be 1.5 for *FGF3* and 4.5 for *HLA* (Table 2 legend). The *FGF3* linkage result is compatible with our previous study of IDDM and chromosome 11q (ref. 12) which concluded that the region did not contain a susceptibility locus with a strong effect, for example a locus similar to *HLA*, but left open the possibility that it contained a locus similar to that discussed here. (The results were expressed in terms of the probability, P_2 , that affected siblings shared two

TABLE 1 Number of families and affected offspring in IDDM family panels

Affected offspring	Initial screen	Number of families	
		Other families	
		Both parents	Parent(s) missing
2	39	123	42
3	2	15	3
4	1	2	2
5	1	1	0
Total families	43	141	47
Total affected sibpairs	61	190	63

All diabetics were ketone-positive at diagnosis and required daily insulin treatment. Patients were aged 45 or younger at onset, or had islet cell autoantibodies, and were of Caucasian descent. Of the families, 133 were of French origin, 22 were of North African origin and 76 were from North America (including 63 available through the Coriell Institute). A staged screening strategy was applied in which markers were initially examined in a sub-panel of 61 affected sibpairs from 20 French families and 23 American families. IBD frequencies were estimated from counts of the number of informative allele pairs (N), and the number that were IBD (I). When both parents were heterozygous and the parental origin of all the alleles could be determined, each affected sibpair contributed two informative allele pairs (one from each parent). Only alleles from the heterozygous parent were counted when the other parent was homozygous. Data have been excluded for infrequent instances when the parents were heterozygous but have identical genotypes (the parental origins of all alleles cannot be determined), or when a parental genotype was not determined for experimental reasons. The IBD probability for a locus was estimated as I/N , and the statistic $T = (I - N/2)/N^{1/2}$ was used to test for linkage. The value of the statistic was compared with tables of the standardized normal distribution to obtain a one-sided significance level. The results are given without weights for differently sized families for reasons discussed in ref. 20, although the application of the weights suggested by Hodge²¹ does not change the conclusions. When the value of the statistic exceeded 1.64 ($P < 0.05$ in a one-sided test), other affected sibpairs were examined to determine whether linkage could be confirmed at a greater level of significance, such as $P < 0.0001$. When the 251 affected sibpairs with DNA available from both parents were studied, the results were analysed as described above. When data on families with missing parents are included, the analysis method is as described in Table 2 legend. Power calculations show that the probability of detecting linkage ($P < 0.05$) in the sub-panel, and confirming ($P < 0.0001$) with the remaining sibpairs, is greater than 90% for a susceptibility locus with an IBD > 65%, using the index map described in Fig. 1 (unpublished results).

alleles with IBD at the locus. We excluded loci for $P_2 > 0.5$, and we observe $P_2 = 0.34$ at *FGF3*.)

Moreover, *FGF3* resides outside the region of conserved synteny with mouse chromosome 9 that was the focus of our previous study. In the mouse, *FGF3* and the linked marker *PYGM* are assigned to chromosomes 7 and 19, respectively, in regions not known to contain susceptibility loci in the NOD mouse or the BB rat^{7,8}. In the human, several potential candidate genes have been mapped near *FGF3*¹³. These include *MDU1*, encoding a cell-surface protein involved in the regulation of intracellular

calcium¹⁴, and *ZFMI*, encoding a putative nuclear protein¹⁵ (both expressed in the pancreas). *RT6*, also in the region, encodes a T-cell membrane protein¹⁶ for which defective expression exists in the BB rat and NOD mouse^{17,18}, although it is not linked to diabetes in the BB rat¹⁹.

In addition to the above results, our study suggests that the MHC contains the predominant genetic susceptibility factors for IDDM. Although we cannot exclude the presence of a second major susceptibility locus linked to *HLA* on chromosome 6p, our results (see Table 1 legend) make it unlikely that a locus

TABLE 2 Analysis of markers showing potential linkage in IDDM-affected sibpairs

a, Markers for which the test of linkage gave $P < 0.05$ for at least one category of diabetics when characterized for 251 affected sibpairs

Chr	Marker	Locus symbol	All IDDM			DR3 IDDM			DR4 IDDM		
			1	0	T	1	0	T	1	0	T
1q	AFM046xh10	D1S191	164	132	1.86	66	62		109	71	2.83*
1q	AFM031xd12	D1S412	160	127	1.95	74	62		114	80	2.44*
4q	AFM304wb9	D4S1604	118	104		65	45	1.91	82	72	
6p	HLA	HLA	298	99	9.99†						
8p	AFM143xd8	D8S264	195	153	2.25	83	71		120	96	1.71
8p	AFM127xh2	D8S262	134	107	1.73	84	69		65	61	
8p	AFM198wd2	D8S277	157	115	2.54*	76	49	2.41*	96	67	2.27
10p	AFM289zd1	D10S582	175	139	2.03	90	66	1.92	108	91	
11p	AFM077xe1	D11S903	173	148		90	63	2.18	112	105	
11q	AFM211xe1	D11S1313	164	146		94	52	3.46†	101	90	
11q	PYGM	PYGM	189	152	2.00	100	58	3.34†	119	101	
11q	AFMa131ye5	D11S987	180	153		100	68	2.56*	119	109	
11q	FGF3	FGF3	173	128	2.59*	95	46	4.12†	98	77	
11q	AFM185ya1	D11S916	192	148	2.38*	104	59	3.52†	118	92	
11q	AFM238xe7	D11S1321	126	91	2.37*	69	42	2.56*	80	73	
11q	AFM155xh10	D11S911	187	150	2.01	91	59	2.61*	123	100	
11q	AFM256zb5	D11S937	194	176		101	74	2.04	117	117	

b, Genotype and allele IBD, and test statistics for *FGF3* in all diabetics and in non-overlapping DR subgroups of diabetic sibpairs

	Genotype IBD			Allele IBD		Test statistics for allele IBD
	2	1	0	1	0	
All IDDM sibpairs	40 (34%)	59 (50%)	20 (16%)	173 (57%)	128 (43%)	2.59 ($P = 0.0048$)
Both DR3/4	14 (47%)	14 (47%)	2 (7%)	48 (66%)	25 (34%)	2.69 ($P = 0.0036$)
Other DR3 sharing	12 (44%)	13 (48%)	2 (7%)	47 (68%)	21 (32%)	3.15 ($P = 0.0008$)
Other DR4 sharing	9 (23%)	21 (54%)	9 (23%)	50 (49%)	52 (51%)	0.0 (NS)
Not sharing DR3 or DR4	5 (22%)	11 (48%)	7 (30%)	28 (48%)	30 (52%)	0.0 (NS)

c, Allele IBD and test statistics for *FGF3* in diabetic sibpairs that have two *HLA* alleles IBD, and 1 or 0 *HLA* alleles IBD

Genotype IBD at <i>HLA</i>	Allele IBD at <i>FGF3</i>		Test statistic
	1	0	
Pairs sharing 2 <i>HLA</i> alleles IBD	72	46	2.70 ($P = 0.0034$)
Pairs sharing 1 or 0 <i>HLA</i> allele IBD	53	48	0.25 (NS)

d, Linkage analysis using estimated IBD probabilities for five loci in the region of *FGF3* on chromosome 11q

Marker	Locus symbol	T-statistic and P value	
		All diabetics	DR3 diabetics
AFM211xe1	D11S1313	1.37 (NS)	2.25 ($P = 0.0130$)
PYGM	PYGM	3.40 ($P = 0.0004$)	3.47 ($P = 0.0003$)
AFMa131ye5	D11S987	2.26 ($P = 0.0124$)	2.13 ($P = 0.0174$)
FGF3	FGF3	3.20 ($P = 0.0008$)	4.02 ($P < 0.0001$)
AFM185ya1	D11S916	2.74 ($P = 0.0032$)	3.36 ($P = 0.0005$)

a, IBD data and test statistics (when $P < 0.05$) for markers that were characterized in 251 affected sibpairs when at least one of the three test statistics exceeded the 0.05 critical value. b, Results for IBD at *FGF3* for affected sibpairs that are both DR3/4, both DR3 (excluding both DR3/DR4), both DR4 (excluding both DR3/DR4), and other sibpairs. A contingency table analysis of differences in the IBD frequencies in these four groups gave $\chi^2_3 = 14.8$ ($P = 0.002$). The pairs were considered as if they were from independent families for this analysis. Proportions of the sibling recurrence risk that could be attributed to *FGF3* and *HLA* (data not shown) were calculated following Risch⁶ as $0.25/\text{Prob}(\text{sharing } 0 | \text{ both affected})$. For *FGF3*, the denominator is the genotype IBD probability of sharing 0 alleles from the first line of the table. c, IBD at *FGF3* for affected sibpairs divided by genotype IBD at the *HLA* locus. Only families for which four different parental alleles were distinguished at *HLA* are included. d, Results of the linkage tests combining data from families with and without DNA on both parents. The program SIBPAL from the SAGE package²² was used to estimate IBD probabilities from all the family information for each affected sibpair, and to do tests against the expectation of 0.5 under the null hypothesis. This method requires allele frequencies for estimating the IBD probabilities with missing parental data. Gene frequency estimates were obtained for the five markers shown in the table using data on one member of each family, and assuming Hardy-Weinberg equilibrium. Although inappropriate allele frequencies may introduce spurious evidence for linkage with this approach, this should be independent of disease status (that is, if positive results are due to inappropriate gene frequencies, the apparent evidence for linkage should also be found when unaffected sibpairs and pairs in which one sibling is affected and the other unaffected are analysed). We examined 259 pairs of unaffected siblings, and 535 pairs containing one affected and one unaffected offspring from the same families as a control for our results. The tests for linkage were non-significant in these groups.

* $P < 0.01$; † $P < 0.001$; ‡ $P < 0.0001$ in one-sided test. Other values of T shown are significant at $P < 0.05$ when the statistic is presented in the table.

with effects similar to *HLA* exists elsewhere in the genome. Screening in additional families would be useful to determine whether other susceptibility loci with smaller effects are undetected. For example, linkage to the insulin gene region was not seen here, although it is known to contain a susceptibility factor; larger panels would be needed to detect this locus, which has an IBD of <55% (refs 4, 5). Outside *HLA* and the *FGF3* region, the strongest evidence for linkage in the total 251 affected sibpair panel was found with markers on chromosome 8 ($P < 0.005$; Table 2a and Fig. 2). Finally, our results suggest that genetic linkage studies using dense sets of highly informative microsatellite markers will aid the localization of susceptibility genes for other multifactorial disorders. □

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Rapid GDP release from $G_{s\alpha}$ in patients with gain and loss of endocrine function

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LUTEINIZING hormone stimulates testicular Leydig cells to produce testosterone by binding to a receptor that activates the G protein G_s and adenylyl cyclase. Testotoxicosis is a form of precocious puberty in which the Leydig cells secrete testosterone in the absence of luteinizing hormone, often due to constitutive activation of the luteinizing hormone receptor and (indirectly) G_s (refs 1–4). Here we study two unrelated boys suffering from a paradoxical combination of testotoxicosis and pseudohypoparathyroidism type Ia (PHP-Ia)⁵, a condition marked by resistance to hormones acting through cyclic AMP (parathyroid hormone and thyroid-stimulat-

ing hormone) as well as a 50% decrease in erythrocyte G_s activity (the remaining 50% is due to the normal G_s allele)^{5,6}. In both patients, a mutation in the gene encoding the G_s α -subunit replaced alanine at position 366 with serine⁵. We show that this α_s -A366S mutation constitutively activates adenylyl cyclase *in vitro*, causing hormone-independent cAMP accumulation when expressed in cultured cells, and accounting for the testotoxicosis phenotype (as cAMP stimulates testosterone secretion). Although α_s -A366S is quite stable at testis temperature, it is rapidly degraded at 37 °C, explaining the PHP-Ia phenotype caused by loss of G_s activity. *In vitro* experiments indicate that accelerated release of GDP causes both the constitutive activity and the thermolability of α_s -A366S.

Hormone-activated receptors normally activate G_α proteins by catalysing release of tightly bound GDP, which is rapidly replaced by GTP; the protein is again inactivated when its intrinsic GTPase activity converts GTP to GDP. Inhibition of this GTPase inactivation reaction is responsible for all known human diseases caused by mutations in GTPases (including both Ras and α_s) apart from PHP-Ia (refs 7–9). By contrast, our experiments show that the A366S mutation in α_s causes both testotoxicosis and PHP-Ia by affecting the other regulatory step in the GTPase cycle—that is, by causing accelerated release of GDP from the mutant protein.

The steady-state rate of GTP hydrolysis (k_{ss}) by recombinant α_s -A366S was 20 times greater than that of wild-type α_s (Fig. 1a), ruling out the possibility that the mutation inhibits hydrolysis. The k_{ss} of wild-type α_s was comparable to previously reported values, whereas the value for the mutant protein (3.5 min^{-1}) resembled previously reported rate constants ($k_{cat, GTP}$) for single turnover GTP hydrolysis by α_s (4.5 ± 0.4 and $3.2 \pm 0.4 \text{ min}^{-1}$ for the short and long forms of α_s , respectively¹⁰). The thermolability of α_s -A366S unfortunately precluded measurement of its $k_{cat, GTP}$.

We assessed the fractional rate of GDP release ($k_{diss, GDP}$) by measuring the rate at which GDP bound to recombinant proteins was replaced by [³⁵S]GTP- γ S, a hydrolysis-resistant analogue of GTP (Fig. 1b). This rate was 80-fold higher for α_s -A366S (14 min^{-1}) than for wild-type α_s (0.18 min^{-1}). Thus, like activated hormone receptors, the mutation accelerates GDP dissociation, and indeed makes GTP hydrolysis the rate-limiting step in the mutant protein's GTPase cycle ($k_{cat, GTP} \approx 4 \text{ min}^{-1}$), rather than dissociation of GDP ($k_{diss, GDP} \approx 14 \text{ min}^{-1}$).

This indicates that, like receptors, the mutation should increase the fraction of α_s molecules in the GTP-bound active form. To confirm this, we determined the steady-state fractional occupancy of the protein by GTP (Fig. 1c). The ratio of $\alpha_s \cdot \text{GTP}$ to total α_s (measured as bound radioactivity in the presence of [γ -³²P]GTP and [α -³²P]GTP, respectively) was 0.05 for wild-type α_s and 0.65 for the mutant protein.

This increase should cause the mutant protein to activate adenylyl cyclase constitutively in the presence of GTP. In the presence of GTP- γ S, the wild-type and mutant proteins activated adenylyl cyclase identically over α_s concentrations from 0 to 100 nM (Fig. 1d), showing that the mutation does not impair the protein's ability to assume an active conformation and interact productively with its effector. In the presence of GTP (but not in the presence of GDP- β S, a GDP analogue), α_s -A366S constitutively stimulated adenylyl cyclase, while wild-type α_s did not (Fig. 1e), matching their respective steady-state occupancies by GTP. These properties of α_s -A366S explain the gain-of-function phenotype, testotoxicosis, in patients carrying the mutation.

The testes are generally 3.5–5.0 °C cooler than the body¹¹. If α_s -A366S were stable only at the lower temperature of the testis, this would explain the loss-of-function phenotype, PHP-Ia, seen in the same patients; this idea proved to be correct. We stably transfected wild-type or mutant α_s into a cell line derived from Leydig cells (TM3). Immunoblots showed that the cellular content of wild-type α_s was the same at 33 °C and 37 °C, whereas that of α_s -A366S was markedly higher at 33 °C (Fig. 2a). Similar

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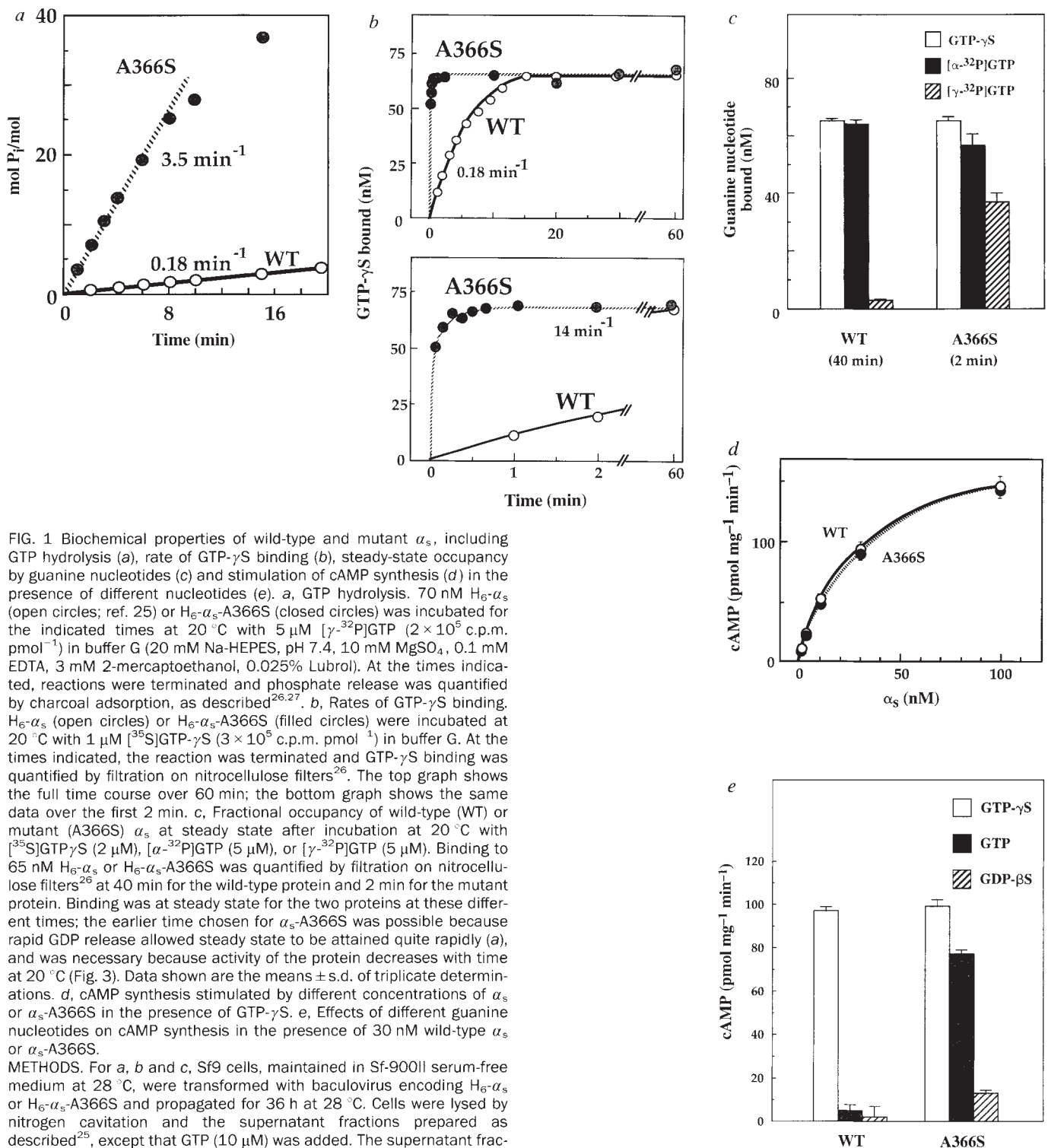


FIG. 1 Biochemical properties of wild-type and mutant α_s , including GTP hydrolysis (a), rate of GTP- γ S binding (b), steady-state occupancy by guanine nucleotides (c) and stimulation of cAMP synthesis (d) in the presence of different nucleotides (e). **a**, GTP hydrolysis. 70 nM $\text{H}_6\text{-}\alpha_s$ (open circles; ref. 25) or $\text{H}_6\text{-}\alpha_s$ -A366S (closed circles) was incubated for the indicated times at 20 °C with 5 μM $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ ($2 \times 10^5 \text{ c.p.m. pmol}^{-1}$) in buffer G (20 mM Na-HEPES, pH 7.4, 10 mM MgSO_4 , 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 0.025% Lubrol). At the times indicated, reactions were terminated and phosphate release was quantified by charcoal adsorption, as described^{26,27}. **b**, Rates of GTP- γ S binding. $\text{H}_6\text{-}\alpha_s$ (open circles) or $\text{H}_6\text{-}\alpha_s$ -A366S (filled circles) were incubated at 20 °C with 1 μM $[\gamma\text{-}^{32}\text{P}]\text{GTP-}\gamma\text{S}$ ($3 \times 10^5 \text{ c.p.m. pmol}^{-1}$) in buffer G. At the times indicated, the reaction was terminated and GTP- γ S binding was quantified by filtration on nitrocellulose filters²⁶. The top graph shows the full time course over 60 min; the bottom graph shows the same data over the first 2 min. **c**, Fractional occupancy of wild-type (WT) or mutant (A366S) α_s at steady state after incubation at 20 °C with $[\gamma\text{-}^{32}\text{P}]\text{GTP-}\gamma\text{S}$ (2 μM), $[\alpha\text{-}^{32}\text{P}]\text{GTP}$ (5 μM), or $[\gamma\text{-}^{32}\text{P}]\text{GTP}$ (5 μM). Binding to 65 nM $\text{H}_6\text{-}\alpha_s$ or $\text{H}_6\text{-}\alpha_s$ -A366S was quantified by filtration on nitrocellulose filters²⁶ at 40 min for the wild-type protein and 2 min for the mutant protein. Binding was at steady state for the two proteins at these different times; the earlier time chosen for α_s -A366S was possible because rapid GDP release allowed steady state to be attained quite rapidly (a), and was necessary because activity of the protein decreases with time at 20 °C (Fig. 3). Data shown are the means $\pm$ s.d. of triplicate determinations. **d**, cAMP synthesis stimulated by different concentrations of α_s or α_s -A366S in the presence of GTP- γ S. **e**, Effects of different guanine nucleotides on cAMP synthesis in the presence of 30 nM wild-type α_s or α_s -A366S.

METHODS. For **a**, **b** and **c**, Sf9 cells, maintained in Sf-900II serum-free medium at 28 °C, were transformed with baculovirus encoding $\text{H}_6\text{-}\alpha_s$ or $\text{H}_6\text{-}\alpha_s$ -A366S and propagated for 36 h at 28 °C. Cells were lysed by nitrogen cavitation and the supernatant fractions prepared as described²⁵, except that GTP (10 μM) was added. The supernatant fractions in buffer A (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 2 mM MgCl_2 , 100 mM NaCl, 10% glycerol, 1 mM 2-mercaptoethanol, 4 μM GTP, 0.1 mM phenylmethylsulphonyl fluoride, 1 $\mu\text{g ml}^{-1}$ pepstatin A, 4 $\mu\text{g ml}^{-1}$ leupeptin and 8 $\mu\text{g ml}^{-1}$ aprotinin) was applied to a column of HiTrap Q (Pharmacia; 5 ml bed volume) which had been equilibrated with 25 ml buffer A. The column was washed with 10 ml buffer A and eluted at a flow rate of 2.0 ml min^{-1} with a 24-ml linear gradient of NaCl (100–220 mM) in buffer A. Both $\text{H}_6\text{-}\alpha_s$ and $\text{H}_6\text{-}\alpha_s$ -A366S were recovered at 160 mM NaCl. Fractions containing α_s were then applied to a column of HiTrap Chelating (Pharmacia; 1-ml bed volume), charged with Ni^{2+} , which had been equilibrated with modified buffer A (lacking EDTA and containing 300 mM NaCl and 10 mM imidazole-HCl, pH 7.4). The column was washed with the modified buffer A and then eluted at a flow rate of 0.5 ml min^{-1} with a 10-ml linear gradient of imidazole-HCl (10–300 mM) in the same buffer. α_s was recovered at 200 mM imida-

zole-HCl. Purified samples were desalted by repeated dilution and concentration (using Centricon-30 filters, Amicon) in buffer A containing 50 mM NaCl and stored at -80 °C. Wild-type and mutant protein concentrations were determined by binding of $[\gamma\text{-}^{32}\text{P}]\text{GTP-}\gamma\text{S}$, as described in **c**. For **d**, reactions were done at 20 °C for 10 min in 50- μl volumes containing 15 μg cyc^- membranes, as described²⁸ apart from minor changes in buffer conditions, including: 50 mM Na-HEPES (pH 8.0), 6 mM MgCl_2 , 0.01% Lubrol and 50 $\mu\text{g ml}^{-1}$ bovine serum albumin. Before the assay, the α_s proteins were incubated with 100 μM GTP- γ S for 30 min (wild type) or 5 min (mutant). For **e**, reactions were for 10 min, as described above, in the presence of 30 nM wild-type α_s or α_s -A366S. Before the assay, α_s or α_s -A366S was incubated with GTP- γ S, GTP or GDP- β S, each at 100 μM .

TABLE 1 Denaturation rate correlates with frequency of the α_e state

	α_e frequency (min^{-1})*	k_{loss} (min^{-1})†	α_e frequency/ k_{loss}
α_s -wild type + GTP or GDP	0.18	0.0011	163
α_s -A366S + GTP	3.5	0.023	152
+ GDP	14	0.087	160

* The frequency with which α_s passes through the empty (α_e) state (and therefore the concentration of α_e) depends on what step is limiting in the GTPase cycle (Fig. 1a). For α_s -A366S in the presence of GTP, hydrolysis of GTP is the slow step in the cycle, with the result that α_e frequency is equal to the steady-state GTPase rate, k_{ss} (Fig. 1c). For α_s -A366S in the presence of GDP, the α_e state recurs every time GDP dissociates, so that α_e frequency is equal to the rate constant for GDP dissociation, $k_{\text{diss,GDP}}$. In the case of wild-type α_s in the presence of GTP, $k_{\text{diss,GDP}}$ limits the rate at which the cycle turns over (that is, k_{ss}); in the presence of GDP, the α_e frequency must be equal to $k_{\text{diss,GDP}}$. Thus for wild-type α_s , the α_e frequency is the same, whether GDP or GTP is present, because $k_{\text{ss}} \approx k_{\text{diss,GDP}}$ (Fig. 1b). Note that this analysis assumes extremely rapid and equal rates of association of GTP and GDP with α_e ; variations in the duration of the α_e state would otherwise produce different rates of denaturation.

† Derived from denaturation curves in Fig. 3.

results were obtained in S49 *cyc*⁻ lymphoma cells (results not shown). cAMP levels in TM3 cells expressing α_s -A366S were 3- to 4-fold higher at 33 °C than at 37 °C (Fig. 2b), whereas expression of wild-type α_s did not elevate cAMP levels above those in untransfected cells at either temperature (data not shown). After expression as a stable protein at 32 °C, α_s -A366S disappeared almost completely from *cyc*⁻ cells within 5 h of being shifted to 37 °C (Fig. 2c); the half-life of the mutant protein at this temperature was less than one hour, as opposed to the wild-type half-life of 21 h (ref. 12).

If this rapid degradation causes the PHP-Ia phenotype, then it should be possible to treat these patients' testotoxicosis by warming the testis to body temperature, as this would induce destruction of the mutant α_s , reducing testosterone secretion.

To investigate the mechanism by which the A366S mutation destabilizes α_s , we compared the stabilities of wild-type and mutant recombinant α_s at 20 °C in the presence of different guanine nucleotides under conditions in which no proteolysis was observed. As was the case in intact cells, α_s -A366S was much less stable than wild-type α_s (Fig. 3). 10 μM GTP stabilized α_s -A366S much better than the same concentration of GDP (Fig. 3 and Table 1). A low concentration of guanine nucleotide (0.5 μM GTP) accelerated denaturation of both proteins, although the mutant denatured more rapidly than wild type (Fig. 3a, b). AlF_4^- ion, which can substitute for the γ -phosphate of GTP in other G_α proteins¹³, also stabilized the GDP-bound mutant protein (Fig. 3c).

Like dissociation of bound GTP- γS , denaturation of wild-type or mutant α_s bound to GTP- γS is extremely slow, indicating that tight binding of GTP or its analogues stabilizes α_s . GDP dissociates much more slowly from the wild type than from the mutant protein, and stabilizes it much better, suggesting that α_s -GDP, like α_s -GTP, resists denaturation. The form of α_s most susceptible to denaturation is thus the transient empty state (α_e) between the dissociation of GDP and the association of GTP (the nucleotide-exchange step of the GTPase cycle), in keeping with observations^{14,15} that exogenous nucleotides stabilize G_α . Because the γ -phosphate of GTP (or AlF_4^- plus GDP) strongly inhibits both nucleotide release and α_s denaturation, denaturation in the presence of GTP probably entails its hydrolysis to GDP followed by transit through α_e .

If denaturation occurs primarily in α_e , then the rate of denaturation should be proportional to the concentration of α_e , which in turn is proportional to the frequency with which the protein enters this state (under conditions noted in Table 1). Quantita-

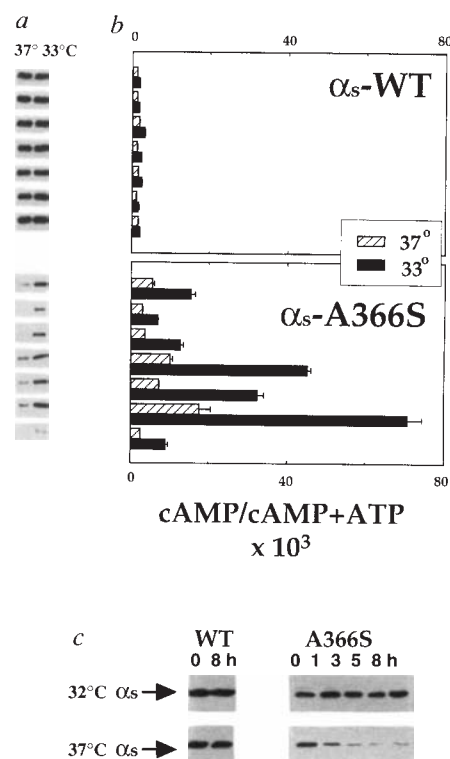


FIG. 2 Expression of recombinant protein (a) and cAMP accumulation (b) in TM3 cells transfected with wild-type or mutant α_s and effect of temperature on expression of the same proteins in S49 *cyc*⁻ lymphoma cells (c). a, Expression of HA- α_s or HA- α_s -A366S in TM3 cells. Proteins from clones of cells cultured at 37 and 33 °C were detected by immunoblotting with monoclonal antibody 12CA5, after immunoprecipitation with the same antibody¹². b, cAMP accumulation in TM3 cells. For each clone of transfected TM3 cells, cross-hatched and filled bars indicate cAMP accumulated for 30 min in cells cultured at 37 and 33 °C, respectively. Values shown represent means $\pm$ s.d. of triplicate determinations. The cAMP increase at 37 °C in cells transfected with mutant as opposed to wild-type α_s cDNAs indicates that a small amount of mutant α_s persisted in cells at this temperature and was able to stimulate adenyl cyclase; a parallel persistence of mutant α_s protein and elevated cAMP in tissues of the patients (outside the testes) would have caused symptoms mimicking hormone excess, rather than PHP-Ia. This discrepancy presumably results from lower expression of endogenous α_s genes (wild-type and mutant) in the patients' cells than in cultured cells transfected with recombinant cDNAs; in the latter case expression was driven by a strong retroviral promoter rather than by the natural α_s promoter (see below). c, Effect of culture temperature on expression of α_s or α_s -A366S in S49 *cyc*⁻ cells. Cells transfected with HA- α_s or HA- α_s -A366S were cultured at 32 °C for 24 h; cultures were then divided and aliquots were cultured separately at 37 or 32 °C. At the times indicated, 2×10^7 cells were withdrawn and protein was determined by immunoblotting. METHODS. TM3 cells were maintained in DMEM/F-12 containing 2.5% fetal calf serum and 5% horse serum. Cells were transfected with the retroviral vector pMV7 containing either WT or mutant α_s DNA as described¹². S49 *cyc*⁻ cells were clones transfected with wild-type or mutant α_s as described¹².

tive analysis shows this is so (Table 1). Denaturation thus appears to stem from the same molecular mechanism that accelerates GDP release from α_s -A366S.

The alanine residue at position 366 is located in a highly conserved short stretch of amino acids called G-5 (refs 16, 17). The three-dimensional structure of this region (including the alanine residue at the position corresponding to 366) is preserved in the α -subunit of retinal transducin (α_t)^{18,19}, Ras^{20,21} and elongation factor Tu²². In all these structures, G-5 forms a loop whose residues stabilize the guanine ring of GTP or GDP either by

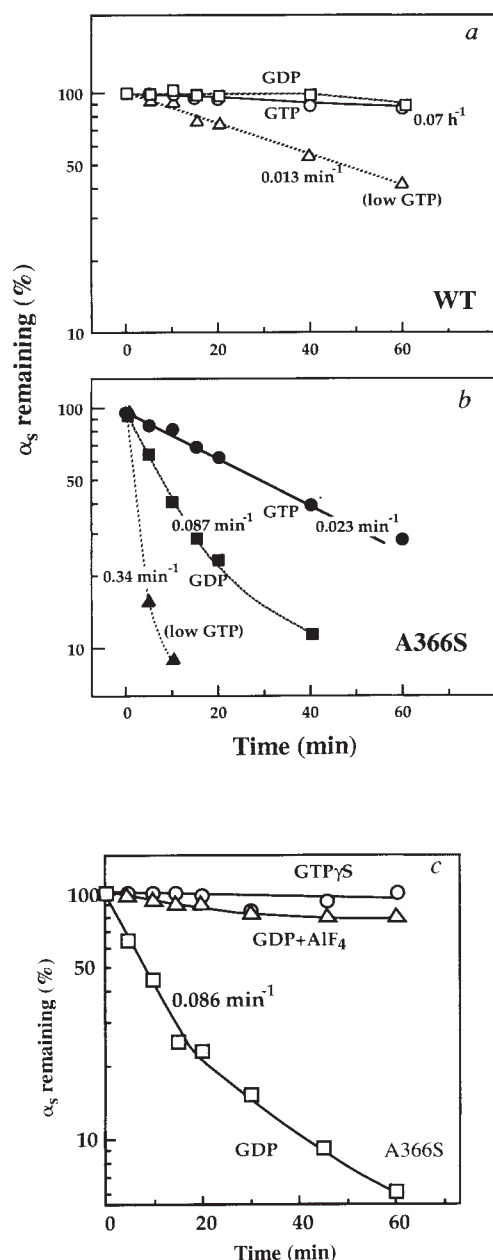


FIG. 3 Stability of recombinant α_s in vitro, assessed by ^{35}S GTP- γS binding (a, b) or ability to stimulate adenyl cyclase (c). Wild-type (a) or mutant (b) α_s (each at 70 nM) was incubated at 20 °C with the indicated concentrations of GTP or GDP. At the times indicated, 5- μl aliquots were withdrawn and assayed for GTP- γS binding in 20- μl reaction mixtures (Fig. 1 legend). For both proteins, loss of activity *in vitro* was caused by denaturation, rather than by proteolysis, in that neither the wild-type nor mutant protein changed in apparent molecular size or concentration, as assessed by SDS-PAGE and immunoblotting with an anti- α_s antipeptide antiserum¹², under any of these incubation conditions (result not shown). c, Stability of recombinant α_s -A366S in vitro, assessed by its ability to stimulate adenyl cyclase. α_s -A366S (70 nM) was incubated at 20 °C with GTP- γS (circles, 10 μM), GDP (squares, 10 μM), or 10 μM GDP with 20 μM AlCl₃ and 10 mM NaF (triangles). At the times indicated 20- μl aliquots were withdrawn, incubated with 100 μM GTP- γS for 5 min (20 °C), and assayed for stimulation of adenyl cyclase in 50- μl reaction mixtures, as described in the legend for Fig. 1d, e. Remaining concentrations of α_s -A366S (per cent of control) were calculated from the relative adenyl cyclase activities (not shown), using the concentration-response curve of Fig. 1d. Note that we used stimulation of adenyl cyclase, rather than binding of radioactive GTP- γS , to estimate the stabilizing effects of GTP- γS and AlF₄ because both treatments markedly reduced the rate of subsequent binding of ^{35}S GTP- γS .

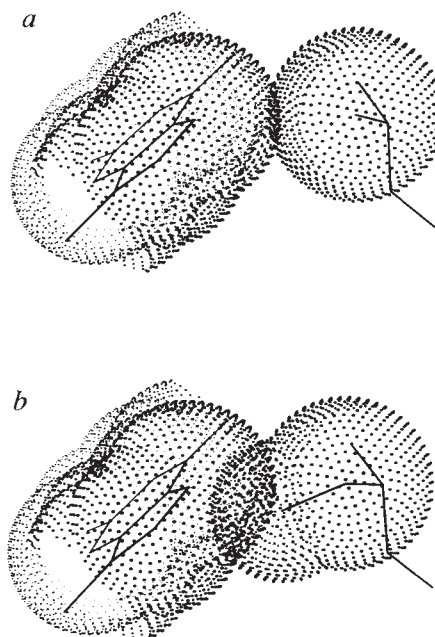


FIG. 4 Postulated interactions of the guanine ring with alanine compared with serine at position 366 of α_s . N7 of the guanine ring makes a van der Waals contact with the methyl group of alanine (a) but clearly overlaps with the methol group of serine (b). Structures shown are based on coordinates of α_1 -GTP- γS (ref. 18), kindly provided by P. Sigler; the underlying assumption is that the guanine ring interacts with the side chain of Ala 366 in α_s just as it interacts with the side chain of Ala 322 in α_1 .

direct van der Waals contacts or by interactions with other residues that make such contacts.

As depicted in Fig. 4, the methyl side chain of the corresponding alanine in α_1 -GTP- γS (A322) makes a van der Waals contact with N7 of the guanine ring. Substitution of a serine for the alanine produces a 2-Å overlap with N7, indicating that such a substitution may displace the guanine ring, disrupting the co-operative network of hydrogen bonds surrounding it.

The GDP-releasing effect of the A366S mutation raises the possibility that receptors catalyse GDP release by acting on the G-5 region. Site-directed mutations in the G-5 regions of other GTPases also reduce GDP-binding affinity. A C325A mutation in α_o , homologous to C365 in α_s , reduces GDP affinity ~10-fold²³. Similarly, replacement of the alanine in Ras homologous to A366 in α_s produces effects resembling those described here, namely a 1,000-fold increase in the rate of guanine-nucleotide exchange, accompanied by constitutive activity leading to neoplastic transformation of cells expressing the mutant protein²⁴. □

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Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate

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THE pleckstrin homology (PH) domain is a new protein module of around 100 amino acids found in several proteins involved in signal transduction^{1–5}. Although its specific function has yet to be elucidated, the carboxy-terminal regions of many PH domains bind to the $\beta\gamma$ subunits of G proteins^{6,7}. On the basis of structural similarities between PH domains and lipid-binding proteins, we have proposed that PH domains may be binding to lipophilic molecules⁸. Indeed, many of the proteins that contain this domain associate with phospholipid membranes^{6,9,10}, and disruption of this domain can interfere with membrane association^{6,11}. Here we report that PH domains bind to phosphatidylinositol-4,5-bisphosphate and show that the lipid-binding site is located at the lip of the β -barrel. This suggests that PH domains may be important for membrane localization of proteins through interactions with phosphatidylinositol-4,5-bisphosphate.

The binding of PH domains to phospholipid vesicles was examined in a centrifugation assay. The amino-terminal PH domain of pleckstrin is unable to bind to many of the phospholipids examined (Fig. 1a), but did bind to phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) in phosphatidylcholine vesicles (Fig. 1a) in a concentration-dependent fashion (Fig. 1b) as well as to PtdIns(4,5)P₂ in other lipid backgrounds (data not shown). In addition, phosphatidylinositol-4-phosphate (PtdIns(4)P) exhibits measurable binding to the PH domain, albeit with a lower apparent affinity (Fig. 1b), and high levels of phosphatidylserine and phosphatidic acid (>25% vesicle components) display some affinity for the PH domains (data not shown). As a control, binding of other proteins to PtdIns(4,5)P₂ was examined. Except for the PH domains, none of the proteins exhibits any binding affinity to vesicles containing PtdIns(4,5)P₂ (Fig. 1c). In contrast, all the PH domains bind, including the C-terminal PH domain of pleckstrin as well as PH domains from T-cell-specific kinase (Tsk), rasGAP and the β -adrenergic receptor kinase (β -Ark) (Fig. 1c).

To investigate the site of interaction of the PH domains with PtdIns(4,5)P₂, the ¹H, ¹³C and ¹⁵N chemical shifts of the N-terminal PH domain of pleckstrin were followed as a function of added lipid (Fig. 2). On addition of PtdIns(4,5)P₂, systematic chemical shift changes were observed for signals corresponding

to residues K13, K14, S16, V17, N19, T20, W21, K22, F35, Y36 and G46. All these residues are located in the N-terminal half of the PH domain at the lip of the β -barrel (Fig. 2). Lysines 13, 14 and 22, as well as T20, W21, F35, Y36 and G46, are conserved in many of the PH domains^{2–5}. These results suggest that the positively charged lysines at the entry of the β -barrel may bind to the negatively charged phosphates of PtdIns(4,5)P₂. Further evidence of the importance of the lysines was obtained in binding studies of protein containing acetylated lysines. In these experiments the acetylated PH domain (Ac-PH) was unable to bind to the phospholipid vesicles containing PtdIns(4,5)P₂ (data not shown). Involvement of the PtdIns(4,5)P₂ phosphates in the binding interaction is supported by the changes in the ³¹P NMR signals of PtdIns(4,5)P₂ on addition of the PH domain. As shown in Fig. 3, the phosphates at the 4,5 positions of the inositol ring exhibit the greatest changes in chemical shift during titration with PH domain. Addition of lysozyme, FK506-binding protein (FKBP), or the Ac-PH domain of pleckstrin, however, resulted in no chemical shift changes for any PtdIns(4,5)P₂ phosphates. Analysis of the changes in ³¹P chemical shifts of PtdIns(4,5)P₂ on addition of PH domain gave an apparent dissociation constant of ~30 μ M. Addition of the N-terminal PH domain of pleckstrin to both PtdIns(4)P and phosphatidylinositol-3-phosphate (PtdIns(3)P) also resulted in ³¹P chemical shift changes, indicating that these lipids bind to PH domains (data not shown). These results suggest that the binding of PtdIns(4,5)P₂ to the PH domains is mediated primarily by ionic interactions. However, the apparent inability of InsP₃ to inhibit the binding of PtdIns(4,5)P₂ to PH domains at similar molar concentrations (Fig. 4) suggests that other interactions may help stabilize the complex. Although the lipophilic residues of the β -barrel of the PH domain could also contribute to the binding through interactions with the fatty acid chains of PtdIns(4,5)P₂ (ref. 8), no major chemical shift changes were detected for residues located deep in the hydrophobic core of the β -barrel.

What is the functional relevance of the interaction between the PH domains and PtdIns(4,5)P₂? Most of the proteins that contain PH domains need to be associated with membranes for their function. But many of these proteins do not contain the classical membrane-anchoring groups such as a hydrophobic helix or sites for post-translational addition of lipid molecules. For example, the C-terminal region of β -spectrin, which contains a PH domain^{4,9}, has been shown to interact with brain membranes depleted of peripheral proteins⁹, but does not contain a classical membrane-anchoring group. The Src family of tyrosine kinases, which are targeted to cellular membranes, contain a consensus sequence for myristylation in their N-terminal regions¹². Three members of a subfamily of tyrosine kinases with extensive homology to the Src family (the Tec kinase from haematopoietic cells¹³, Tsk from T cells¹⁴, and Bruton's tyrosine kinase (Btk) from B and myeloid cells¹⁵) do not have an N-terminal myristylation site or any other candidate for membrane interaction. However, they do contain a PH domain at the N terminus⁴. Another kinase that contains a PH domain, β -Ark, must also be localized at the membrane surface in order to phosphorylate its receptor substrate effectively^{6,16}. Although removal of the last 128 residues of β -Ark (essentially the entire PH domain) decreases the kinase activity to basal level, replacement of this domain with an isoprenylation site restores most of this activity⁶. A homologous protein, rhodopsin kinase, lacks the C-terminal PH domain of β -Ark, but contains an isoprenylation site instead¹⁷. Additional evidence for the functional correlation of PH domains and membrane localization was found in studies of phospholipase C- δ 1 (PLC- δ 1). In this protein the N-terminal region containing the PH domain is necessary for binding to phospholipid vesicles containing PtdIns(4,5)P₂, and this binding is necessary for processive catalytic activity¹¹. All these observations suggest that PH domains may have a biologically significant role in the localization of proteins to phospholipid membranes.

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Lefkowitz and co-workers have shown that several PH domains bind to the $\beta\gamma$ subunits of G proteins^{6,7}. In these studies, the C-terminal region of the PH domain and residues extending beyond the C terminus of this module were required for binding^{6,7}. Our results indicate that the N terminus of the PH domain is responsible for binding to PtdIns(4,5)P₂, but they do not exclude the possibility that the PH domains also bind to

$\beta\gamma$ subunits. Indeed, the binding of the N-terminal portion to PtdIns(4,5)P₂ may localize the protein to the membrane, thus facilitating the interaction of the C-terminal portion with $\beta\gamma$ subunits.

PtdIns(4,5)P₂ is a source of the important second messengers inositol trisphosphate and diacylglycerol, and in the past 10 years the importance of lipids as precursors of bioactive

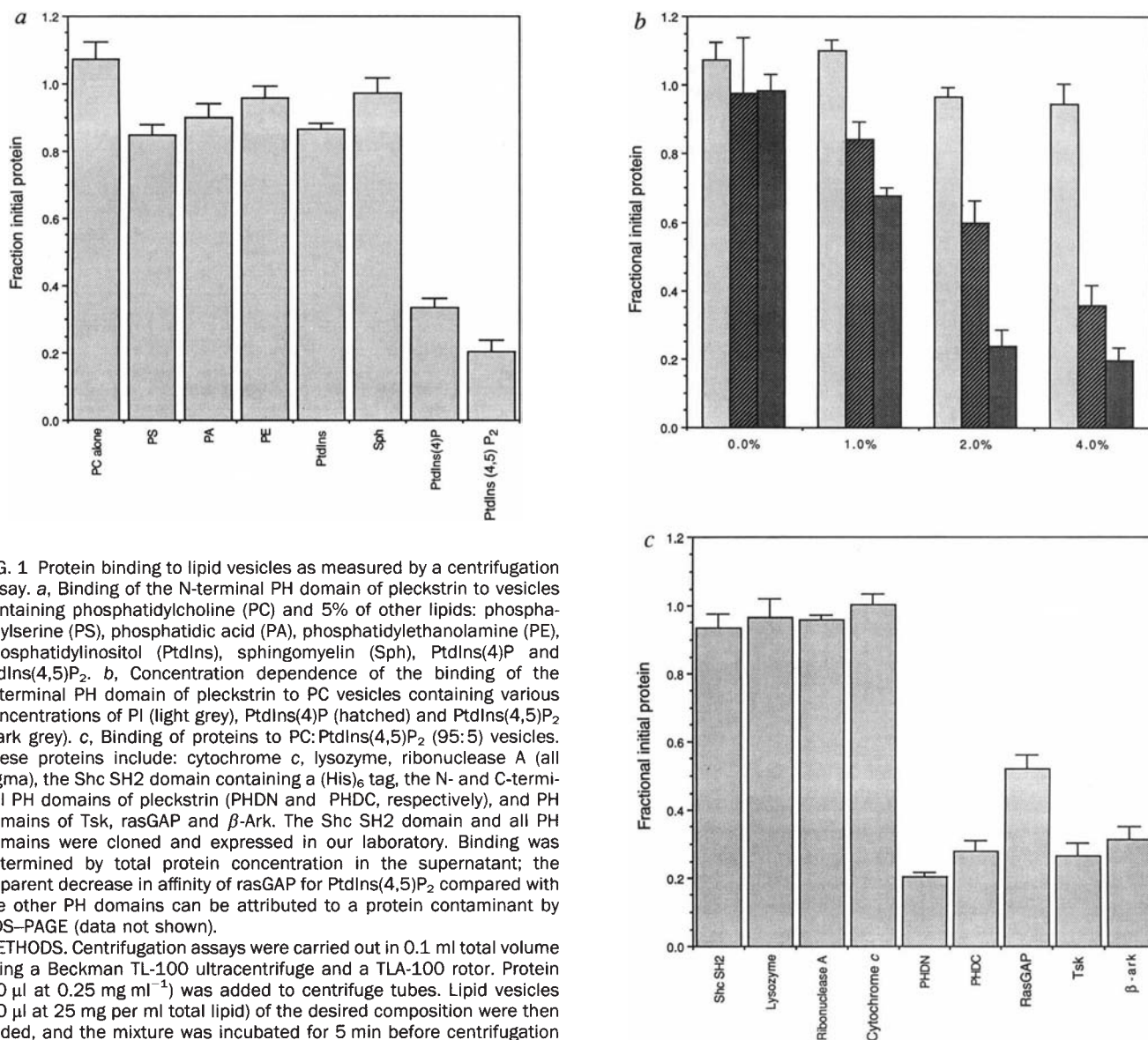


FIG. 1 Protein binding to lipid vesicles as measured by a centrifugation assay. **a**, Binding of the N-terminal PH domain of pleckstrin to vesicles containing phosphatidylcholine (PC) and 5% of other lipids: phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylinositol (PtdIns), sphingomyelin (Sph), PtdIns(4)P and PtdIns(4,5)P₂. **b**, Concentration dependence of the binding of the N-terminal PH domain of pleckstrin to PC vesicles containing various concentrations of PI (light grey), PtdIns(4)P (hatched) and PtdIns(4,5)P₂ (dark grey). **c**, Binding of proteins to PC:PtdIns(4,5)P₂ (95:5) vesicles. These proteins include: cytochrome c, lysozyme, ribonuclease A (all sigma), the Shc SH2 domain containing a (His)₆ tag, the N- and C-terminal PH domains of pleckstrin (PHDN and PHDC, respectively), and PH domains of Tsk, rasGAP and β -Ark. The Shc SH2 domain and all PH domains were cloned and expressed in our laboratory. Binding was determined by total protein concentration in the supernatant; the apparent decrease in affinity of rasGAP for PtdIns(4,5)P₂ compared with the other PH domains can be attributed to a protein contaminant by SDS-PAGE (data not shown).

METHODS. Centrifugation assays were carried out in 0.1 ml total volume using a Beckman TL-100 ultracentrifuge and a TLA-100 rotor. Protein (50 μ l at 0.25 mg ml⁻¹) was added to centrifuge tubes. Lipid vesicles (50 μ l at 25 mg per ml total lipid) of the desired composition were then added, and the mixture was incubated for 5 min before centrifugation at 100,000 g for 30 min. The vesicles pelleted with this treatment, removing any bound protein from the supernatant. The protein concentrations of the supernatants (50 μ l) was then determined by the BCA protein assay (Pierce, Rockford, Illinois) and compared to that of protein not exposed to lipid. Phospholipid blanks were run to correct for any background due to phospholipid in the protein assay. Vesicles were formed by probe sonication of the lipid suspension for several minutes. Both protein and lipid suspensions were in 10 mM K-PO₄ pH 7.4, 100 mM NaCl, and 2.7 mM KCl (PBS). All PH domains in this study were cloned from poly(A)⁺ RNA using reverse transcriptase-PCR. The amplified region and the source of poly(A)⁺ RNA for each PH domain were as follows: the N-(M1-G105) and C-terminal (E240-K350) PH domains of pleckstrin were from poly(A)⁺ RNA of HL60 promyelocytic leukemia cell line, TSK (M1-R111) was from Jurkat T-cell line poly(A)⁺ RNA, and the Ras-GAP (A470-R579) and β -Ark (G556-A654) were from human placenta poly(A)⁺ RNA. Complementary DNAs were then

subcloned into pET20b plasmid and expressed in *Escherichia coli* (HMS174(DE3) or BL21(DE3)) cells, and all except the β -Ark PH domain contained an additional Leu-Glu-(His)₆ sequence at the C terminus. All proteins containing the Leu-Glu-(His)₆ sequence were purified by affinity chromatography on a nickel-IDA column (Invitrogen) and exchanged into PBS. The β -Ark PH domain was expressed within inclusion bodies which were washed with Tris buffer (50 mM Tris-HCl, 50 mM NaCl buffer, pH 7.0) three times. The second and third washes also contained 0.2% dodecylmaltoside (C₁₂M), and 0.32% C₁₂M plus 1 M NaCl respectively. The washed inclusion bodies were solubilized in PBS containing 6 M urea, 20 mM dithiothreitol (DTT) and subjected to chromatography on Superdex 75. The purified β -Ark PH domain was then refolded by dialysis against decreasing amounts of urea in PBS with 20 mM DTT. The refolded protein was exchanged into PBS without DTT before assay.

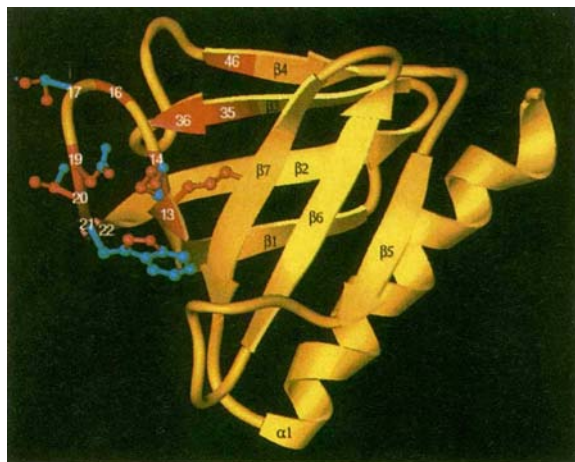


FIG. 2 Ribbon plot¹⁹ of the N-terminal PH domain of pleckstrin. Residues whose backbone resonances (N, HN, C α or H α) showed significant chemical shift changes on addition of PtdIns(4,5)P₂ are shown in red. Also shown in red are side chain heavy atoms for which the heteroatom and attached proton(s) showed significant chemical shift changes (side chain heavy atoms shown in blue either did not shift or could not be followed as a result of signal overlap). Chemical shift changes were considered significant if (1) they were systematic as a function of PtdIns(4,5)P₂ concentration, and (2) the magnitude of the change was greater than twice the digital resolution of the spectra (0.005, 0.04 and 0.05 p.p.m. for ¹H, ¹⁵N and ¹³C, respectively).

METHODS. All NMR spectra were acquired at 298 K on a Bruker AMX 600 NMR spectrometer. All samples were in exchanged into phosphate buffer (20 mM, pH = 6.5) containing 100 mM NaCl and 5 mM DTT. The ¹H, ¹³C and ¹⁵N assignments were taken from Yoon *et al.*⁵. The large size of the protein-vesicle complex precluded direct observation of the protein resonances, and the addition of PtdIns(4,5)P₂ alone caused precipitation of the complex. Therefore, titrations were performed in 24.4 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), where the protein, PtdIns(4,5)P₂ and the complex were soluble. The detergent alone had no apparent effect on the chemical shifts of the protein. Titrations were performed on samples of 0.5–1.0 mM protein, and PtdIns(4,5)P₂ was added sequentially out to a maximum of 2.0 molar equivalents.

molecules and second messengers has become widely recognized¹⁸. Our results suggest that particular lipids may also be important for localizing proteins at the membrane surface. The PH domains may offer an alternative vehicle to hydrophobic helices or post-translational addition of lipid molecules for membrane localization. The interaction of PH domains with PtdIns(4,5)P₂ provides a novel mechanism for membrane association and may be critical in controlling the biological functions of the many proteins that contain this module. □

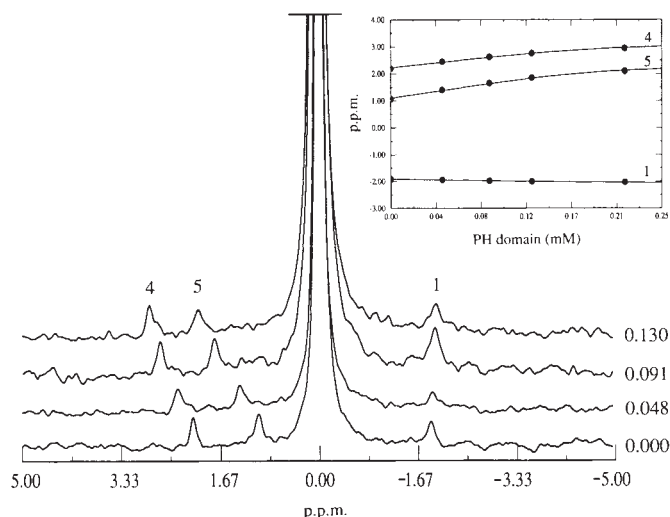


FIG. 3 ³¹P NMR spectra of PtdIns(4,5)P₂ (0.2 mM in 24.4 mM CHAPS buffer as described in Fig. 2) as a function of increasing concentration of the N-terminal PH domain of pleckstrin (final mM protein concentration given to the right of each spectrum). Numbers above the peaks correspond to the phosphates at positions 1, 4, and 5 of the inositol ring of PtdIns(4,5)P₂ (phosphate assignments were obtained by comparing spectra of PtdIns(4,5)P₂, (PtdIns(4)P and PC). Phosphate present in the buffer (large central peak) was referenced to 0.0 p.p.m. The inset shows a fit to the chemical shift data using the following equation: $\delta_{\text{obs}} = \delta_f + \chi_b \Delta_{b-f}$, where δ_{obs} is the observed chemical shift, χ_b is the mole fraction in the bound state, δ_f is the chemical shift of the free state, and Δ_{b-f} is the difference in chemical shift between the free and bound states ($\delta_b - \delta_f$). Mole fractions were calculated from a dissociation constant, K_d , using the known initial concentrations of the protein and PtdIns(4,5)P₂. A least-squares analysis was then performed by systematic variation of K_d , δ_f and Δ_{b-f} . Fitted parameters and estimated errors (in parentheses) for resonances corresponding to positions 4 and 5 were the following: Position 4: $K_d = 32$ (11) μM ; $\delta_f = 2.22$ (0.02) p.p.m.; $\Delta_{b-f} = 0.99$ (0.05) p.p.m. Position 5: $K_d = 23$ (8) μM ; $\delta_f = 1.10$ (0.02) p.p.m.; $\Delta_{b-f} = 1.29$ (0.05) p.p.m. Fits of data for position 1 were insensitive to K_d because of the small chemical shift changes. Holding K_d constant at 27 μM yielded $\delta_f = -1.91$ (0.02) p.p.m. and $\Delta_{b-f} = -0.16$ (0.05) p.p.m. for position 1.

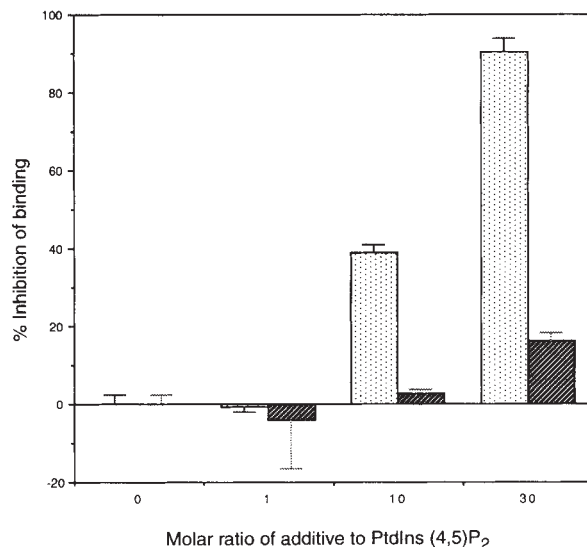


FIG. 4 Inhibition of binding of the N-terminal PH domain of pleckstrin to 98:2 (PC:PtdIns(4,5)P₂) vesicles. The N-terminal PH domain was incubated with the additives for 20 min at room temperature before the addition of the lipid vesicles. Binding assays were performed as described in Fig. 1. Stippled box, InsP₃; hatched box, inositol.

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c-Fos transcriptional activity stimulated by H-Ras-activated protein kinase distinct from JNK and ERK

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Ras proteins exert their mitogenic and oncogenic effects through activation of downstream protein kinases¹. An important question is how Ras-generated signals reach the nucleus to activate downstream target genes. AP-1, a heterodimeric complex of Jun and Fos proteins, which activates mitogen-inducible genes², is a major nuclear target of Ras³. Ras can stimulate AP-1 activity by inducing *c-fos* transcription^{2,3}, a process which is probably mediated by the ERK1 and -2 mitogen-activated protein (MAP) kinases⁴, which phosphorylate the transcription factor Elk-1/TCF^{5,6}. Besides inducing transcription from *fos* and *jun* genes, mitogens and Ras proteins enhance AP-1 activity through phosphorylation of c-Jun^{7,8}. Phosphorylation of the c-Jun activation domain leads to c-Jun induction through an autoregulatory loop². Ras- and ultraviolet-responsive protein kinases that phosphorylate c-Jun on serine residues at positions 63 and 73 and stimulate its transcriptional activity have been identified⁹. These proline-directed kinases, termed JNKs, are novel MAP kinases¹⁰. It is not clear, however, whether c-Jun is the only recipient and JNK the only transducer of the Ras signal to AP-1 proteins. A short sequence surrounding the major JNK phosphorylation site of c-Jun is conserved in c-Fos and is part of its activation domain¹¹, suggesting that c-Fos may be similarly regulated. Here we show that Ras does indeed augment the transcriptional activity of c-Fos through phosphorylation at Thr 232, the homologue of Ser 73 of c-Jun. However, this is mediated by a novel Ras- and mitogen-responsive proline-directed protein kinase that is different from JNKs and ERKs. Therefore, at least three types of proline-directed kinases⁴ transmit Ras- and mitogen-generated signals to the transcriptional machinery.

The sequence surrounding the major JNK phosphorylation site in c-Jun is conserved in c-Fos¹¹, such that Thr 232 is homologous to Ser 73 in c-Jun (Fig. 1a). Both sequences correspond to the HOB1 regions of the Jun and Fos activation domains¹¹. To test whether the c-Fos activation domain is Ras-responsive, the region encoding amino acids 210–313 of c-Fos (containing HOB1 and HOB2) was fused to the DNA-binding domain of GHF-1, a pituitary-specific activator¹² (Fig. 1a). GHF1-cFos(210–313) activated the rGH-LUC reporter and its activity was stimulated 7-fold by oncogenic H-Ras (Fig. 1b). Both basal and Ras-induced activities of GHF1-cFos(210–313) are similar to those of a cJun-GHF1 chimera⁷. The activity of GHF1-cFos(210–313) was also potentiated by incubation of transiently transfected A431 cells with epidermal growth factor (EGF) (Fig. 1c). Substitution of Thr 232 by an alanine residue (T232A) had no effect on the basal activity of GHF1-cFos(210–313; T232A) but abolished its response to H-Ras or EGF. A similar effect was obtained by substitution of Pro 233 by either glycine or valine residues. The activation of GHF1-cFos(210–313) by EGF was Ras-dependent as it was inhibited by the dominant negative¹³ H-ras(Asn17) mutant (Fig. 1c). As H-Ras does not affect GHF-1 DNA-binding activity¹⁴, the enhancement of GHF1-cFos(210–313) activity is due to potentiation of the c-Fos activation function.

The transcriptional activity of c-Jun is stimulated by ultraviolet irradiation¹⁵, which results in strong activation of JNK^{9,10}. However, activation by GHF1-cFos(210–313) was not potentiated by ultraviolet irradiation or 12-*O*-tetradecanoyl phorbol 13-acetate (TPA; Fig. 1b). Hence, despite the similarity between the HOB1 regions of c-Jun and c-Fos, the kinase that phosphorylates Thr 232 is different from the JNKs, whose activity is stimulated more efficiently by ultraviolet irradiation than by H-Ras expression^{9,10}. As TPA is an efficient activator of ERK1 and -2 (ref. 16), they are also unlikely candidates for Thr 232 phosphorylation.

H-Ras potentiates activation by c-Jun homodimers^{7,8} and increases endogenous AP-1 activity¹⁷. By dimerizing with c-Jun, c-Fos is an important contributor to AP-1 activity^{18–20}. We therefore examined whether a c-Jun/c-Fos heterodimer also responds to H-Ras. Transactivation of the AP-1-responsive -73Col-LUC reporter by c-Fos plus c-Jun was indeed stimulated by H-Ras (Fig. 1d). This response is mediated by phosphorylation of both c-Jun and c-Fos, as heterodimers containing one wild-type partner and one mutant partner lacking the Ras-responsive phosphorylation sites, c-Jun(S63A; S73A) or c-Fos(T232A), were partially responsive. But transactivation of -73Col-LUC by c-Fos(T232A) plus c-Jun(S63A; S73A) was no longer H-Ras-responsive. This result suggests that phosphorylation at Thr 232 is also required for H-Ras responsiveness when c-Fos is complexed with its physiological partner c-Jun. Substitution of Thr 232 by alanine and/or H-Ras expression had no effect on accumulation of c-Fos or its interaction with c-Jun (data not shown).

Transient coexpression of oncogenic H-Ras with c-Fos resulted in increased phosphorylation of wild-type (WT) c-Fos but not of c-Fos(T232A) (data not shown). Two-dimensional mapping²¹ of tryptic peptides derived from wild-type c-Fos and c-Fos(T232A) confirmed these observations and indicated that Thr 232 is the major H-Ras-responsive phosphoacceptor of c-Fos (Fig. 2a). Most of the H-Ras-mediated increase in c-Fos phosphorylation was localized to phosphopeptide R, which was absent from digests of c-Fos(T232A). Therefore phosphopeptide R most probably arises from phosphorylation of Thr 232. Furthermore, the migration position of this peptide is entirely consistent with its predicted amino-acid composition and size²¹ (41 amino acids, due to cleavage between Asp 246 and Pro 247 by performic acid²²). This assignment was confirmed by the response of a c-Fos(T232S) mutant to H-Ras (Fig. 2b). Phosphoaminoacid analysis of the Ras-induced phosphopeptide R' revealed the presence of phosphoserine; phosphopeptide R derived from wild-type c-Fos contained phosphothreonine (Fig. 2c). H-Ras expression also led to a slight and variable increase in phosphopeptides r1 and r2, derived from wild-type c-Fos, and decreased the level of others (Fig. 2a, b). These changes were also observed with c-Fos(T232A). Phosphoaminoacid analysis revealed the presence of phosphoserine in both r1 and r2 (data not shown).

By contrast to H-Ras, ultraviolet irradiation and TPA had a marginal effect on c-Fos phosphorylation (Fig. 3a). The small increase in total c-Fos phosphorylation after TPA or ultraviolet exposure was restricted to phosphopeptides 2 and 4, and no changes in phosphopeptide R were detected (data not shown). TPA, however, activated ERK2, whereas ultraviolet irradiation activated JNK1 (data not shown, but see ref. 9). It is therefore unlikely that either the ERKs or the JNKs phosphorylate c-Fos at Thr 232. This assertion is further supported by *in vitro* experiments. Using c-Jun/c-Fos heterodimers as substrates, ERK1/2 (a mixture of both enzymes) phosphorylated wild-type c-Fos and c-Fos(T232A) with comparable efficiency (Fig. 3b). Phosphorylation of c-Fos by ERK1/2 gave rise to phosphopeptides 2 and 4, which are also phosphorylated *in vivo* (Fig. 3c). The low level of c-Jun phosphorylation by ERK1/2 occurs at Ser 243, as previously reported²³. JNK1, on the other hand,

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phosphorylated c-Jun very efficiently but did not phosphorylate c-Fos.

To identify the Ras-responsive protein kinase that phosphorylates Thr 232, we used A431 cells in which c-H-Ras is efficiently activated by EGF²⁴ and the EGF effect on c-Fos transcriptional activity is Ras-dependent (Fig. 1c). As revealed by an in-gel kinase assay²⁵, incubation of A431 cells with EGF resulted in

rapid activation of protein kinase of relative molecular mass 88K, termed FRK (Fos-regulating kinase), which phosphorylated c-Fos or GST-cFos incorporated into the gel (Fig. 4a). The 88K FRK has also been detected in EGF-treated F9, COS, HeLa (data not shown) and PC12 cells (Fig. 4b). In a PC12 derivative containing an inducible H-ras(Asn17) allele²⁶, activation of FRK by EGF was inhibited by induction of H-Ras(Asn17),

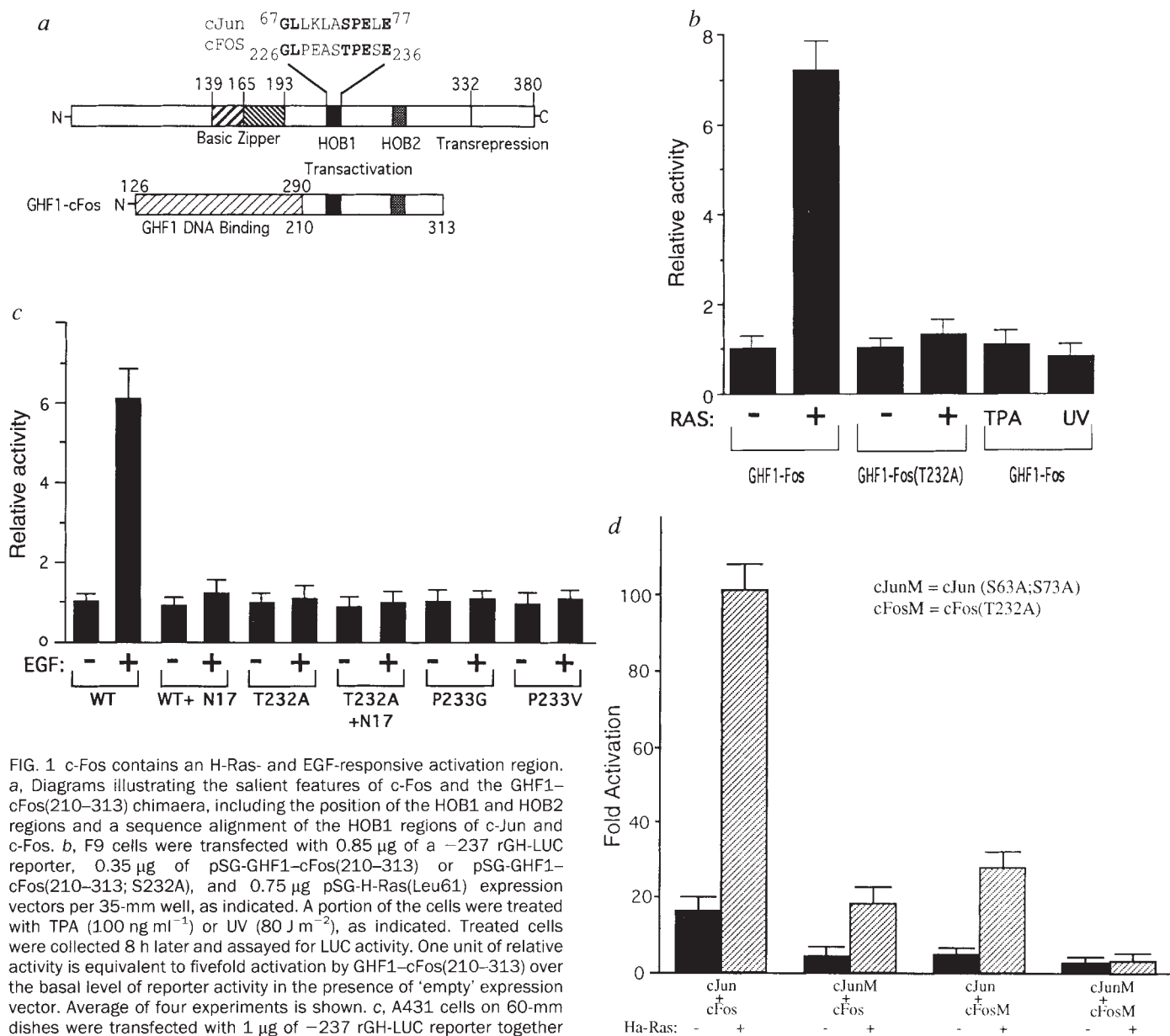


FIG. 1 c-Fos contains an H-Ras- and EGF-responsive activation region.

a, Diagrams illustrating the salient features of c-Fos and the GHF1-cFos(210-313) chimera, including the position of the HOB1 and HOB2 regions and a sequence alignment of the HOB1 regions of c-Jun and c-Fos. **b**, F9 cells were transfected with 0.85 μ g of a -237 rGH-LUC reporter, 0.35 μ g of pSG-GHF1-cFos(210-313) or pSG-GHF1-cFos(210-313;S232A), and 0.75 μ g pSG-H-Ras(Leu61) expression vectors per 35-mm well, as indicated. A portion of the cells were treated with TPA (100 ng ml⁻¹) or UV (80 J m⁻²), as indicated. Treated cells were collected 8 h later and assayed for LUC activity. One unit of relative activity is equivalent to fivefold activation by GHF1-cFos(210-313) over the basal level of reporter activity in the presence of 'empty' expression vector. Average of four experiments is shown. **c**, A431 cells on 60-mm dishes were transfected with 1 μ g of -237 rGH-LUC reporter together with 1 μ g of SR α expression vectors encoding: GHF1-cFos(210-313) and GHF1-cFos(210-313) with T232A, or P233V mutations, using the lipofection procedure³⁵. To examine the involvement of H-Ras in the response to EGF, the SR α -Ras(Asn 17) expression vector (3 μ g) encoding dominant-negative H-Ras¹³ was included as indicated. After 48 h cells were either treated with EGF (100 ng ml⁻¹) or left untreated and collected 8 h later for determination of LUC activity. Average of three experiments is shown. **d**, F9 cells were transfected with 0.3 μ g -73 Col-LUC³⁶ and 0.15 μ g of either RSV-cJun, RSV-cJun(S63A; S73A), pSV40-cFos (mouse) or pSV40-cFos(T232A) with or without 0.3 μ g pSG-H-Ras(Leu 61) per 35-mm well, as indicated. Average of two experiments (each in duplicate) is shown. Fold activation was determined by dividing the actual luciferase activity obtained in the presence of the Jun and Fos expression vectors by the activity of the reporter when cotransfected with an 'empty' expression vector (pRSV-0).

METHODS. To construct GHF1-cFos(210-313), a 1.4-kb HindIII-HpaI fragment from cJ/GHF-1 (ref. 7) was cloned into pSG6 between the HindIII and SmaI sites. pSG6 is derived from pSG5 (ref. 37) and contains

a sequence from the HincII to the BamHI site of pBluescript cloned between the EcoRI and BamHI sites of pSG5. The c-Jun coding region was replaced by the FLAG epitope³⁸. The last two codons and the stop codon of GHF-1 were modified by site-directed mutagenesis to create an NcoI site. The codons encoding AA209/210 and 313/314 of mouse c-Fos were modified by site-directed mutagenesis to generate NcoI and BamHI sites, respectively. The resultant 312-bp NcoI-BamHI fragment containing amino acids 210-313 of mouse c-Fos was inserted between the NcoI site created at the end of the GHF-1 coding region and downstream BamHI site to generate GHF1-cFos(210-313). GHF1-cFos(210-313; T232A) is identical to GHF1-cFos(210-313) except that it contains a Thr→Ala substitution at position 232 introduced by site-directed mutagenesis. Sequences of oligodeoxynucleotides used for mutagenesis are available upon request. The -237 rGH-LUC reporter was made by inserting a 255-bp HindIII-BstYI fragment of rat GH-CAT³⁹ between the HindIII and BamHI sites in the polylinker region of p20LUC³⁶.

further substantiating its identification as a Ras-dependent kinase (Fig. 4b). FRK activity was not detected in the absence of c-Fos or when either c-Jun (data not shown), GST or GST-cFos(T232A) were used as substrates (Fig. 4a). Phosphopeptide mapping indicated that phosphorylation of c-Fos by FRK occurred on peptides R, r1 and r2 (Fig. 4c), the same phosphopeptides detected after H-Ras coexpression. Using full-length c-Fos(T232A) as a substrate, the same kinase activity was detected but phosphorylation occurred only on peptides r1 and r2 (Fig. 4c). Therefore, the EGF-activated 88K FRK is specific for Thr 232 and the two secondary Ras-responsive phosphorylation sites. As substitution of Pro 233 by glycine prevented phosphorylation at Thr 232 (data not shown), FRK is a proline-directed kinase. The occasionally observed EGF-stimulated 60K kinase activity (Fig. 4a) did not phosphorylate c-Fos at Thr 232 (data not shown).

These experiments demonstrate that activated H-Ras augments c-Fos transcriptional activity by stimulating its phosphorylation on Thr 232. The similarity in regulation between c-Jun and c-Fos and the sequences surrounding their H-Ras-stimulated phosphoacceptors suggested the involvement of a

common Ras-activated protein kinase. Contrary to these expectations, we find that the protein kinase that phosphorylates c-Fos at Thr 232 is neither JNK1 (46K) nor JNK2 (55K), which phosphorylate c-Jun at the Ras-responsive sites Ser 63 and 73 (refs 9, 10). This conclusion is based on the inability of ultraviolet irradiation, a potent JNK activator, to induce c-Fos phosphorylation *in vivo* and the failure of immunopurified JNK1 to phosphorylate c-Fos *in vitro*. In addition, TPA, a potent activator of ERK1 and 2 (ref. 16), does not stimulate phosphorylation at Thr 232 *in vivo*, and purified ERK1/2, although they are efficient c-Fos kinases, do not phosphorylate it at Thr 232. Therefore while the mitogen-responsive kinase that phosphorylates c-Fos at Thr 232 is proline-directed, like the JNKs and ERKs, it is a different enzyme. Concurrently, we show that stimulation by EGF results in rapid activation of an 88K protein kinase that phosphorylates c-Fos at Thr 232. In addition to its EGF and Ras responsiveness, like the other MAP kinases, this FRK protein kinase is a proline-directed kinase. Preliminary evidence suggests that the two secondary FRK phosphopeptides (r1, r2) contain Ser 133 and are due to cleavage at Lys 139 and Arg 140, respectively. Serine 133 is also followed by a proline

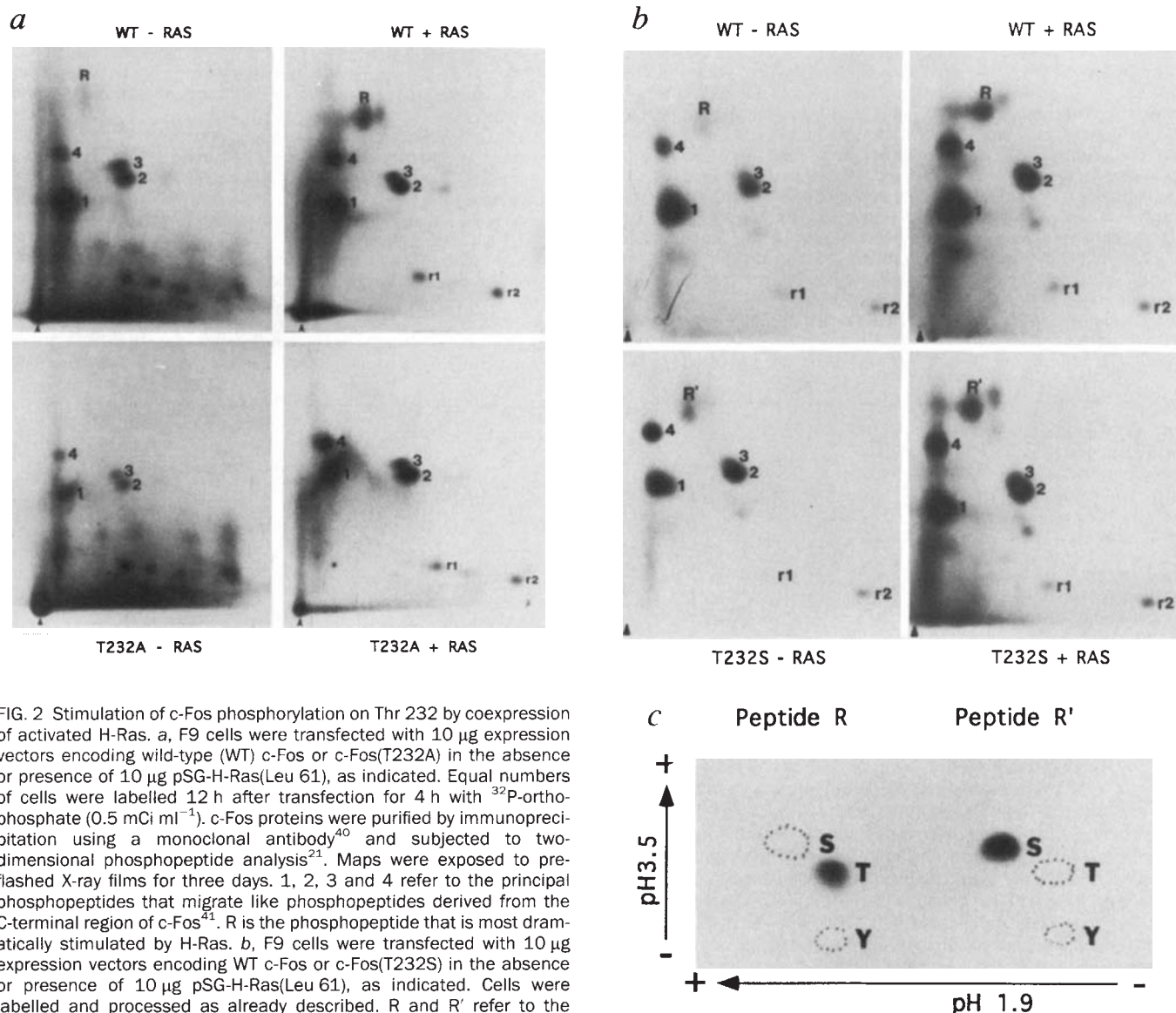


FIG. 2 Stimulation of c-Fos phosphorylation on Thr 232 by coexpression of activated H-Ras. **a**, F9 cells were transfected with 10 μ g expression vectors encoding wild-type (WT) c-Fos or c-Fos(T232A) in the absence or presence of 10 μ g pSG-H-Ras(Leu 61), as indicated. Equal numbers of cells were labelled 12 h after transfection for 4 h with 32 P-orthophosphate (0.5 mCi ml $^{-1}$). c-Fos proteins were purified by immunoprecipitation using a monoclonal antibody⁴⁰ and subjected to two-dimensional phosphopeptide analysis²¹. Maps were exposed to preflashed X-ray films for three days. 1, 2, 3 and 4 refer to the principal phosphopeptides that migrate like phosphopeptides derived from the C-terminal region of c-Fos⁴¹. R is the phosphopeptide that is most dramatically stimulated by H-Ras. **b**, F9 cells were transfected with 10 μ g expression vectors encoding WT c-Fos or c-Fos(T232S) in the absence or presence of 10 μ g pSG-H-Ras(Leu 61), as indicated. Cells were labelled and processed as already described. R and R' refer to the phosphopeptides that are most dramatically elevated in H-Ras-expressing cells and are derived from WT c-Fos and c-Fos(T232S), respectively. **c**, The R and R' phosphopeptides on the chromatograms were extracted from the plates and subjected to partial acid hydrolysis and analysis by two-dimensional high-voltage electrophoresis²¹. 32 P-

labelled phosphoaminoacids were detected by exposure to preflashed X-ray film using an intensifying screen for 8 days at -80°C . Non-radioactive phosphoaminoacid standards were detected by ninhydrin staining.

and is located within a sequence similar to that surrounding Thr 232. Although FRK is the only EGF-activated kinase phosphorylating c-Fos at Thr 232 that can be detected by an in-gel kinase assay, we cannot rule out the existence of other protein kinases with similar properties that are resistant to renaturation.

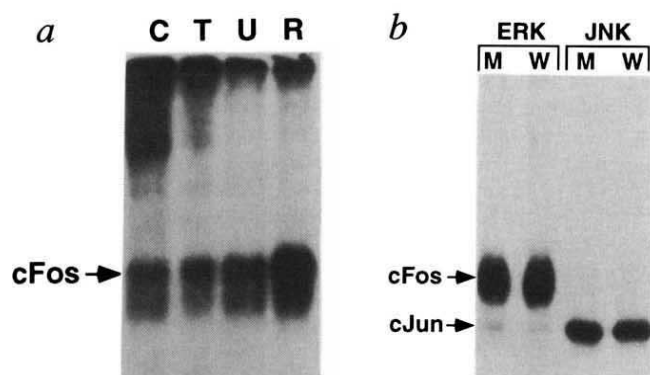
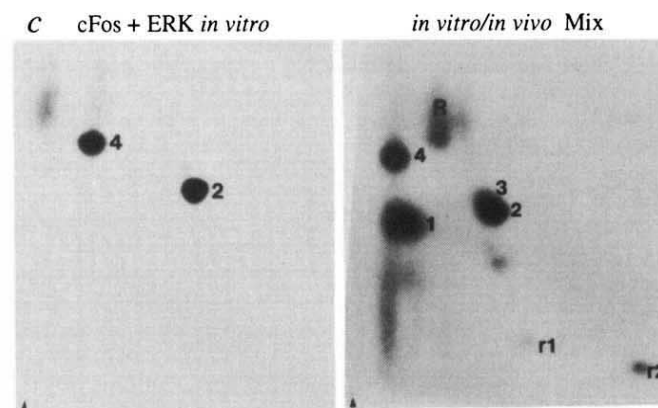


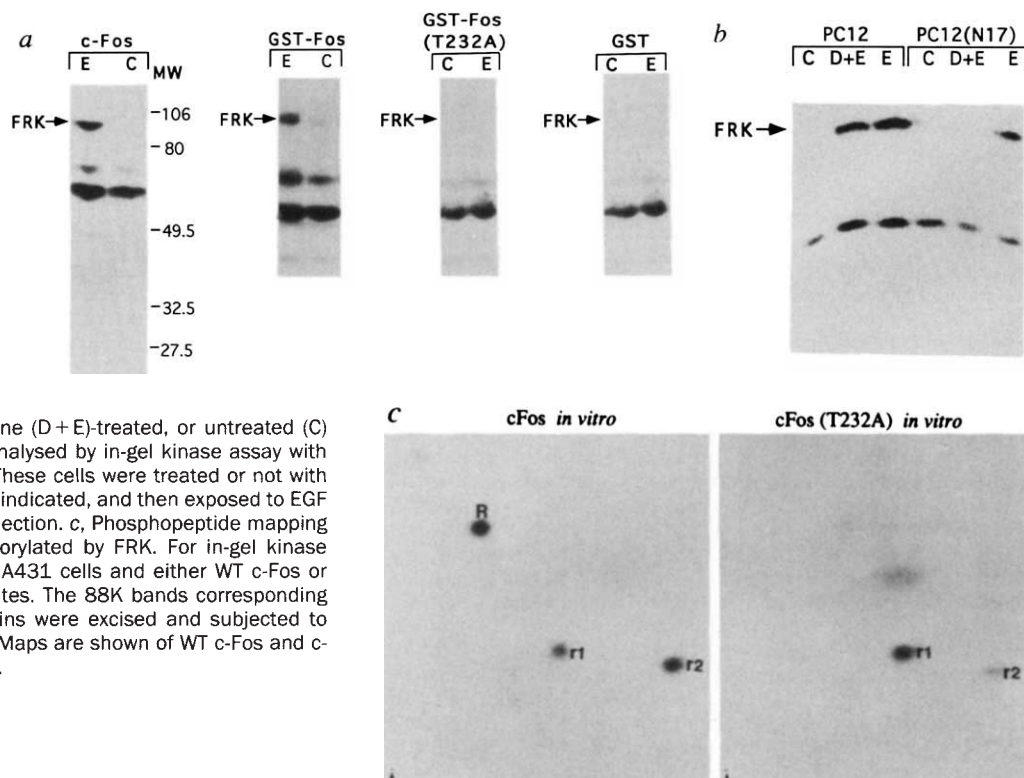
FIG. 3 The kinase that phosphorylates Thr 232 of c-Fos is distinct from JNK and ERK1/2. *a*, *In vivo* phosphorylation of c-Fos. Wild-type c-Fos expression vector was transiently transfected into F9 cells that were treated as indicated. C, control; T, TPA (100 ng ml⁻¹) treatment for 15 min before collecting; U, ultraviolet irradiation (80 J m⁻²), 30 min before collecting; R, cotransfection with pSG-H-Ras(Leu 61), as described for Fig. 2. Cells were labelled and processed as for Fig. 2. c-Fos immunoprecipitates were separated by SDS-PAGE and autoradiographed. *b*, *In vitro* phosphorylation. Recombinant c-Fos or c-Fos(T232A) (50 ng each) were mixed with recombinant c-Jun (40 ng) and incubated at 30 °C for 30 min with immunopurified JNK1 or purified ERK1/2 (a mixture of both enzymes) in kinase buffer containing [γ -³²P]ATP. Reactions were terminated by addition of 6.5 μ l 4 $\times$ SDS-PAGE sample buffer and boiling. W, wild-type c-Fos; M, c-Fos(T232A). Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography. Bands corresponding to c-Jun and c-Fos are indicated. *c*, Two-

dimensional tryptic peptide maps of WT c-Fos phosphorylated *in vitro* by ERK1/2, and a 1:1 mixture between c-Fos phosphorylated *in vitro* by ERK1/2 and c-Fos labelled *in vivo* and isolated from H-Ras-cotransfected cells.



METHODS. Human c-Jun was prepared as described³⁶ and mouse c-Fos (both WT and mutant) was prepared using the same expression system. Both proteins were purified to near homogeneity. The recombinant c-Jun and c-Fos proteins were incubated at 30 °C for 30 min with the relevant kinases in kinase buffer (20 mM HEPES, pH 7.6, 10 mM MgCl₂, 20 mM β -glycerophosphate, 20 mM *p*-nitrophenylphosphate, 0.1 mM NaVO₄, 2 mM DTT) containing 10 μ M [γ -³²P]ATP in 20 μ l. Reactions were terminated by addition of 6.5 μ l 4 $\times$ SDS-PAGE sample buffer and boiling. Purified ERK1/2 is a mixture of both enzymes (gift from M. Cobb). Polyclonal anti-JNK1 antiserum was prepared against recombinant JNK1 and used to immunopurify activated JNK1 from UV-irradiated HeLa cells. Immunoprecipitations were done as described¹⁰.

FIG. 4 Identification of the mitogen-activated kinase phosphorylating c-Fos at Thr 232. *a*, *In-gel* kinase assays. Whole-cell extracts (100 μ g each) of A431 cells treated with EGF (E, 100 ng ml⁻¹ for 15 min) or untreated (C) were separated on SDS-polyacrylamide gels containing immobilized c-Fos, GST-cFos(210–313), GST-cFos(210–313; T232A) or GST proteins. Gels were subjected to renaturation and *in situ* phosphorylation as described⁹. The 88K band corresponding to FRK is indicated. *b*, Whole-cell extracts of EGF-treated (E), EGF plus dexamethasone (D+E)-treated, or untreated (C) PC12[H-Ras(Asn17)] cells²⁶ were analysed by in-gel kinase assay with immobilized c-Fos as a substrate. These cells were treated or not with dexamethasone (10⁻⁶ M) for 3 h as indicated, and then exposed to EGF (100 ng ml⁻¹) for 15 min before collection. *c*, Phosphopeptide mapping of c-Fos and c-Fos(T232A) phosphorylated by FRK. For in-gel kinase assays, extracts of EGF-stimulated A431 cells and either WT c-Fos or c-Fos(T232A) were used as substrates. The 88K bands corresponding to FRK-phosphorylated c-Fos proteins were excised and subjected to tryptic phosphopeptide mapping²¹. Maps are shown of WT c-Fos and c-Fos(T232A) phosphorylated by FRK.



which are absent from the immediate surrounding of Thr 232. Third, RSK phosphorylates c-Fos at Ser 362 (ref. 28), which can be deleted without affecting phosphorylation by FRK.

Although ERK1 and -2 do not enhance c-Jun activity^{9,31}, they are efficient c-Fos kinases (Fig. 3; ref. 28). The major ERK phosphorylation site is Ser 374 of c-Fos²⁸, which accounts for two phosphopeptides²⁸ whose mobilities are similar to those of phosphopeptides 2 and 4 in Fig. 3c. But there is only a small change in phosphorylation of these peptides upon cell stimulation and the functional importance of Ser 374 is unknown²⁸. Despite their small effect on c-Fos phosphorylation, ERK1 and -2 make a very important contribution to the stimulation of AP-1 activity through phosphorylation of Elk-1/TCF, a transcription factor mediating c-fos induction^{5,6}. Thus, once activated, Ras leads to activation of three different types of protein kinases that carry its signal to the nucleus to induce AP-1 activity. The fact that all of these kinases are proline-directed and phosphorylate similar sites in c-Jun, c-Fos and Elk-1/TCF illustrates the complexity of the signalling pathways that transmit information from Ras in the membrane to the transcriptional machinery in the nucleus.

ERK, JNK and FRK differ in their substrate specificities. The molecular mechanisms conferring such a high degree of specificity remain to be determined. Nevertheless, the involvement of three distinct types of proline-directed kinases in transmission of mitogenic signals to the transcriptional machinery may explain why different growth factors that activate very similar sets of signalling molecules^{1,32} can elicit distinct biological responses. This could be mediated through differential deployment of downstream MAP kinases which phosphorylate distinct sets of substrates.

How does increased phosphorylation of c-Jun on Ser 63 and 73 and c-Fos on Thr 232 stimulate their transcriptional activity? One clue to the role of phosphorylation stems from the remarkable conservation between the bipartite activation domains of c-Jun³³ and c-Fos¹¹. It is possible that phosphorylation of sites within the HOB1 region affects its ability to interact with the HOB2 region, resulting in a conformational change that increases the ability of c-Jun and c-Fos to interact with a common target that mediates their activities. Alternatively, phosphorylation of HOB1 may result in recruitment of additional coactivators, which may act in synergy with constitutive coactivators that interact with HOB2. It would be useful to examine the interaction between the c-Fos activation domain and the CBP protein that binds the transcription factor CREB, together with its modulation by phosphorylation, as CBP interacts with c-Jun and potentiates its activation function only after its phosphorylation of Ser 73 (ref. 34). □

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An HMG-like protein that can switch a transcriptional activator to a repressor

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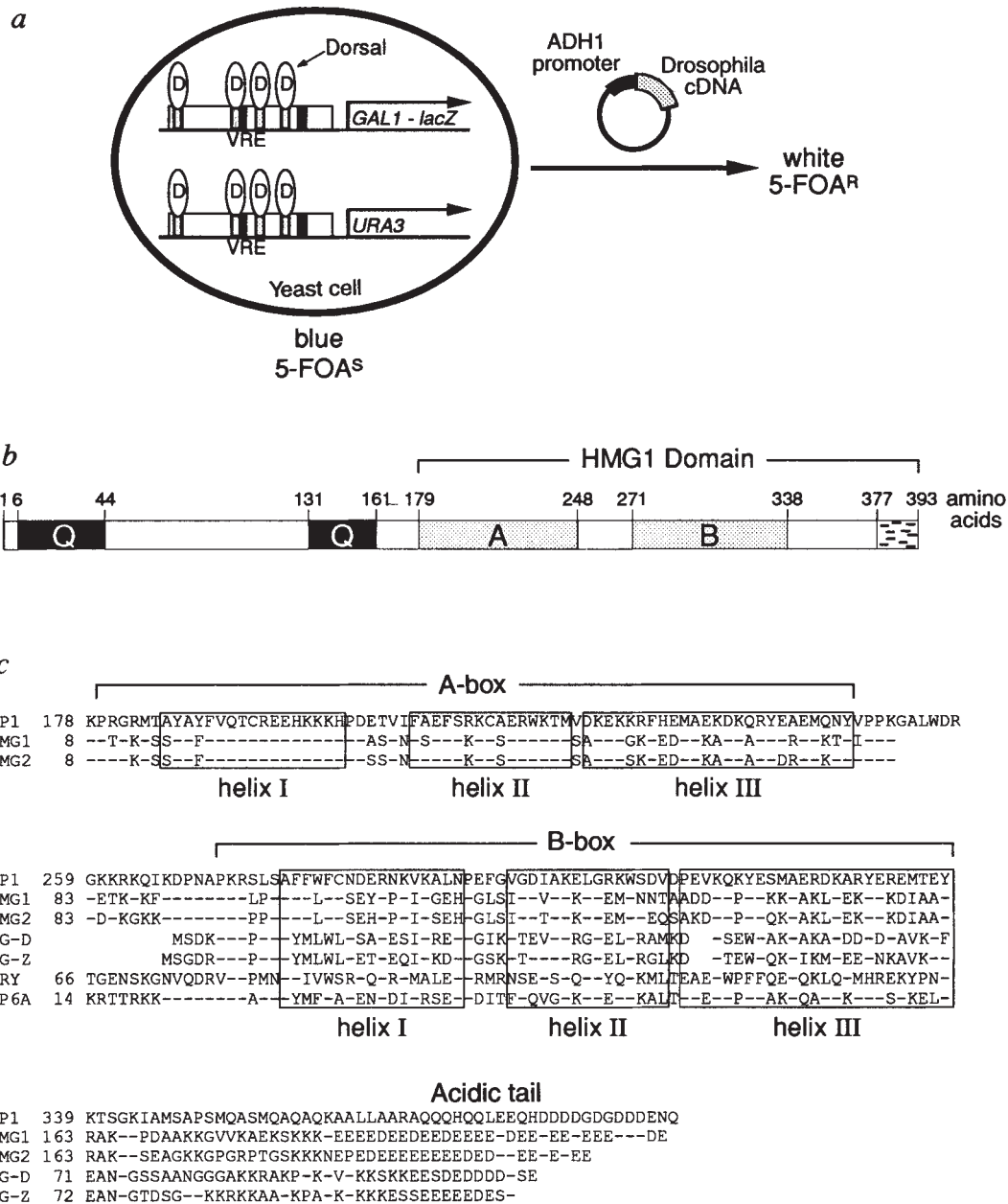
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ONE protein can activate some genes and repress others in the same cell¹. The *Drosophila* protein Dorsal² (which, like the human protein NF- κ B³, is a member of the Rel family of transcriptional activators) activates the *twist* gene and represses the *zen* gene in the ventral region of early embryos^{4,5}. Here we describe a *Drosophila* HMG1 protein, called DSP1 (dorsal switch protein), that converts Dorsal and NF- κ B from transcriptional activators to repressors. This effect requires a sequence termed a negative regulatory element (NRE), found adjacent to Dorsal-binding sites in the *zen* promoter and adjacent to the NF- κ B-binding site in the human interferon- β (IFN- β) enhancer^{6–8}. Previous studies have shown that another type of HMG protein, HMG I(Y), can stimulate NF- κ B activity⁹. Thus, the HMG-like proteins DSP1 and HMG I(Y) can determine whether a specific regulator functions as an activator or a repressor of transcription.

To isolate *Drosophila* complementary DNAs encoding inhibitors of Dorsal, we used the yeast system shown in Fig. 1a. We assumed that the Ventral repression element¹⁰ (VRE) from the *Drosophila zen* promoter contains binding sites (shown in black) for one or more proteins that inhibit activation by Dorsal; such an inhibitor would not affect Dorsal's action on a promoter lacking inhibitor-binding sites. In yeast, Dorsal activates transcription of genes bearing a VRE¹¹, indicating that the putative inhibitor is absent. In the depicted scheme, Dorsal activates both the β -galactosidase reporter gene (staining the yeast colonies blue on the appropriate indicator) and the *URA3* reporter gene (inhibiting yeast growth in the presence of 5-fluoro-orotic acid (5-FOA))¹². We introduced into these cells a cDNA library of early-expressed *Drosophila* genes driven by a strong yeast promoter. Those genes encoding inhibitors could be detected by the formation of white 5-FOA^R colonies. One of these inhibitors worked equally efficiently whether Dorsal activated from sites within the *zen* VRE or from Dorsal-binding sites removed from

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FIG. 1 a, Inhibitor screen. The yeast strain bearing the depicted reporter genes expresses Dorsal under the control of a strong yeast promoter. Each reporter bears upstream a 600-bp fragment taken from the *Drosophila zen* promoter that includes the VRE. The VRE bears binding sites for Dorsal and binding sites (black) for a putative co-repressor as described in the text. The strain was converted to a diploid and used to screen two cDNA libraries derived respectively from 0–4 h and 4–8 h *Drosophila* embryos. Each cDNA was transcribed from the yeast *ADH1* promoter. **b**, Diagram showing various sequence motifs in DSP1. The N-terminal half of the protein contains two glutamine-rich regions denoted by two black boxes labelled 'Q'. The C-terminal half of DSP1 contains an HMG1 domain, consisting of two HMG boxes labelled 'A' and 'B' and an acidic tail (stippled box). The positions of the various motifs within the DSP1 sequence are given by the numbers above the bar diagram. **c**, Sequence alignment of the HMG1 domain of DSP1 with six other members of the HMG1 family. Amino acids identical to DSP1 are marked as -. Gaps in the alignment are left as empty spaces. The positions of three α -helices as found in rat HMG1 are indicated; two human proteins (hHMG1 and hHMG2) bear both A and B HMG boxes; the *Drosophila* proteins HMG-D and HMG-Z, the human protein hSRY, and the yeast protein NHP6A, bear only one (B) box (see ref. 18 for sequences and references). The number on the left presents the position of the first amino acid shown in the native protein. **METHODS.** The *HindIII/XbaI* fragment spanning from -1.6 kb to -1.0 kb in the *zen* promoter (provided by M. Levine) was cloned upstream of a *GAL1/lacZ* fusion and integrated next to the *HIS3* locus of NLY2 (ref. 26) after linearizing with *Afl*II. The *zen* promoter fragment was also cloned upstream of a *PstI/HindIII* fragment containing a promoterless *URA3* gene in puc18. A 5-kb *XbaI* fragment containing the entire *lys2* gene was inserted into the *XbaI* site of this construct. The plasmid was integrated into the *URA3* locus of the strain containing the *zen GAL1/lacZ* fusion after linearizing the plasmid with *Apal* and selecting for the *lys* marker. The plasmid that was used to integrate Dorsal expressed from the constitutive *GPD* promoter has been described previously²⁶. The strain was converted into a diploid using an HO plasmid containing a *leu2* marker (a gift from M. Lamphier). The *Drosophila* cDNA expression libraries were constructed as follows. Two cDNA libraries²⁷ (0–4 h and 4–8 h), provided by N. Brown, were digested with *NotI* and *HindIII*, and ligated directly to the *NotI/HindIII* backbone of pMA1021, a yeast expression vector containing the yeast *ADH1* promoter, the yeast *leu2* marker, and the chloramphenicol resistance gene. The ligation reactions were then transformed into the *Escherichia coli* strain JM101 by electroporation, and plated on media containing chloramphenicol (the vector of the original library did not contain the



chloramphenicol resistance gene). The bacterial transformants were pooled and plasmid DNA was prepared for transformation into yeast strains. Each library was estimated to contain about 2×10^6 clones. Ten micrograms of each library were transformed into the diploid strain by the LiAc method as described previously²⁶ and plated onto 40 plates, each containing medium depleted for leucine, histidine, lysine and tryptophan. This resulted in $\sim 10^6$ transformants for each library, as judged by serial dilutions. After 3 days, the plates were scraped and a 1 in 1,000 dilution of each individual plate was plated onto medium depleted for leucine, histidine, lysine, and tryptophan that contained 0.5 g 5-FOA per litre. After 3 days, a few plates contained as many as 100 colonies, whereas about 10 plates for each library contained no colonies. The plates with colonies were replica plated onto X-gal plates, and the plasmids of the white colonies were rescued and checked for plasmid linkage. Three candidates were isolated from the 4–8 h library, all of which were zygotic *cactus*. Of the 48 candidates obtained from the 0–4 h library, 46 were maternal *cactus* and one was DSP1. pSK-DSP1 was constructed by subcloning DSP1 as a *HindIII/NotI* fragment into pSK+ (Stratagene). DNA sequence of both strands was determined by the dideoxy method with Sequenase (US Biochemicals). Homology to the HMG1 family of DNA-binding proteins was found using the BLAST Network Service at NCBI. The complete nucleotide sequence of DSP1 has been submitted to GenBank, accession number U13881 and is also available upon request.

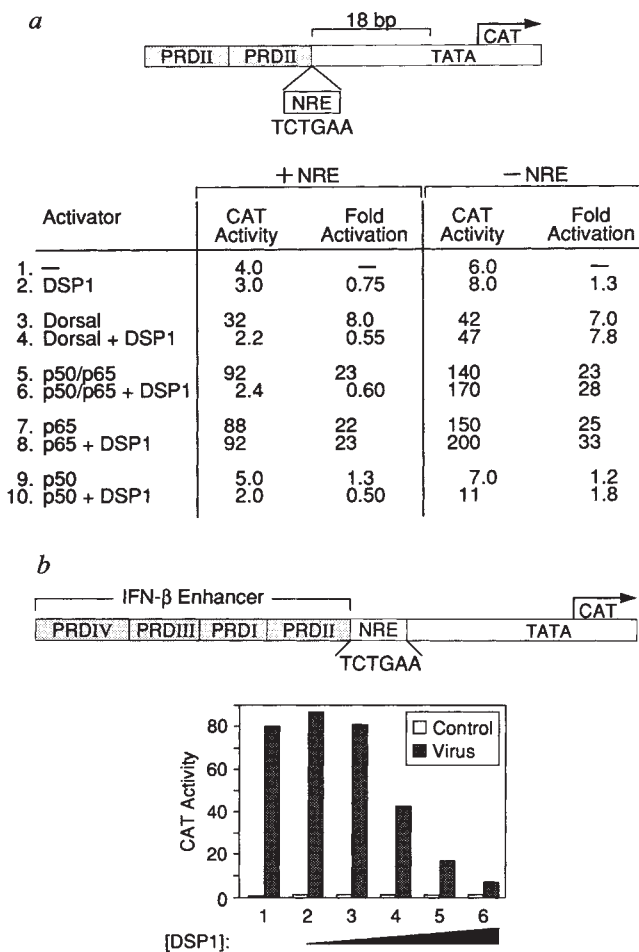


FIG. 2 DSP1 inhibition of Dorsal and NF- κ B. **a**, Reporter plasmids bearing two PRDII sites with (+NRE) or without (-NRE) an NRE upstream of the IFN- β TATA box were cotransfected into HeLa cells along with mammalian expression vectors encoding the indicated Rel family members. These cotransfections were carried out in the presence or absence of the DSP1-expressing plasmid. CAT activity is as described in ref. 20. For each experiment in this and other figures in this paper, the degree of activation is the ratio of CAT activity to that observed in line 1. **b**, DSP1 inhibition of virus induction of the IFN- β enhancer. The reporter plasmid was cotransfected into HeLa cells with increasing amounts of the DSP1 expression plasmid and the cells induced by infection with Sendai virus (shaded bars) or mock induced (white bars). PRDII-IV comprises the IFN- β enhancer. PRDII binds NF- κ B, and the other elements bind other specific activators.

METHODS. An oligonucleotide bearing two copies of the PRDII element adjacent to an NRE was cloned at the *Bam*HI site of the -40IFN β CAT plasmid⁹. This plasmid and the PRDIIICAT plasmid²⁰, which does not contain the NRE, were used in the cotransfection experiments. HeLa cells were transfected by the calcium phosphate method. The transfection cocktail contained 1 μ g of reporter, 1 μ g of CMVLacZ²⁸ used as a transfection efficiency control, 1 μ g of Rel-expressing plasmid and 10 μ g of DSP1 expression vector, or the empty plasmid. Forty-eight hours post-transfection the cells were collected and the chloramphenicol acetyltransferase determined as described⁹. For the experiment shown in **b**, HeLa cells were cotransfected with 6 μ g of -110IFN β CAT reporter plasmid, 3 μ g of CMVLacZ and increasing amounts of DSP1 expression vector (0.5 μ g, 1 μ g, 2 μ g, 4 μ g, 8 μ g). The total amount of DNA was kept constant by including the appropriate amount of empty expression vector. Twenty-four hours post-transfection the cells were either mock- or virus-induced for 18 h and the CAT activity determined as described⁹. The expression plasmids for the mammalian Rel proteins have been described (D.T. and T.M., submitted). pECEdorsal contains the entire coding sequence of Dorsal in pECE72, a derivative of pECE²⁹. pCMV-DSP1 contains the entire coding sequence of DSP1 in pCMV, a mammalian expression vector containing the CMV promoter (Invitrogen).

the VRE context (not shown). The sequence of that candidate inhibitor revealed that it is identical to the previously cloned *cactus* gene^{13,14} (data not shown). In *Drosophila*, the inhibitory protein Cactus sequesters Dorsal in the cytoplasm, and thus acts in a promoter-independent manner¹³⁻¹⁷.

A second inhibitory protein, DSP1, inhibited Dorsal activation of reporter genes bearing the VRE element, but had no effect on reporter genes bearing isolated Dorsal-binding sites, a difference clearly evident by inspection of yeast colony colour (not shown). The level of Dorsal protein in the cells was not affected by expression of DSP1 (not shown). The sequence of DSP1 shows that it is a member of the HMG1 protein family¹⁸, containing two sequence motifs called 'HMG boxes' (A and B)¹⁹ (Fig. 1b, c). Similar motifs direct DNA binding in several eukaryotic proteins¹⁹. Figure 1c compares sequence elements of DSP1 with related sequences. The DSP1 gene lies in region 14CD of the *Drosophila* X chromosome (data not shown). DSP1 messenger RNA is evenly distributed throughout the preblastoderm embryo, and is undetectable after cellularization (as expected if the gene is maternally expressed; data not shown).

DSP1 inhibited activation by Dorsal in mammalian (HeLa) cells (Fig. 2a). The reporter is a gene bearing the NF- κ B-binding site taken from the IFN- β gene enhancer called positive regulatory domain II (PRDII)²⁰. As expected from the fact that the binding sites for Dorsal and NF- κ B are similar and in some cases identical, Dorsal efficiently activated the reporter (Fig. 2a, line 3). This activation was unaffected by the presence of an NRE adjacent to the PRDII sites (Fig. 2a). Cotransfection of an additional construct encoding DSP1 virtually abolished activation of those templates bearing an NRE adjacent to the PRDII sites, but was without effect on templates lacking an NRE (Fig. 2a, line 4).

DSP1 also inhibited activation by NF- κ B in HeLa cells, provided that an NRE is present as shown in Fig. 2a. NF- κ B is a

heterodimer of subunits p50 and p65, and cotransfection with DNA encoding both components activated transcription (Fig. 2a, line 5). DSP1 abolished the effect if the template bore an NRE (Fig. 2a, line 6). Both the p65 and p50 subunits of NF- κ B can bind PRDII as homodimers, and the following experiments showed that inhibition by DSP1 required the presence of p50. Thus, p65 homodimers activated transcription, but this activation was unaffected by DSP1 (Fig. 2a, lines 7 and 8) as was activation by p65/cRel dimers (data not shown). In contrast, heterodimers of p50 and RelB or cRel activated transcription, and this activation was abolished by DSP1 if the template bore an NRE (data not shown). Homodimers of p50 do not activate from PRDII sites (Fig. 2a, line 9; D.T. and T.M., submitted for publication), and we show below that p50 homodimers are converted to repressors by DSP1 in the presence of an NRE. Figure 2b shows that high levels of DSP1 block virus induction of the IFN- β gene enhancer, a regulatory element which binds several different activators in addition to NF- κ B²⁰⁻²². These results are consistent with previous studies showing that the activity of the IFN- β enhancer can be inhibited by blocking any one of these activators²⁰⁻²².

Figure 3 shows that, in the presence of DSP1, various Rel proteins worked as repressors when bound upstream of activators that turn on the thymidine kinase (TK) gene in HeLa cells. This effect is observed only with templates bearing a wild-type NRE. In the experiments shown in Fig. 3a and b, two PRDII elements associated with an NRE are positioned in opposite orientations adjacent to the TK regulatory elements. In Fig. 3c, the NRE is mutant, and in Fig. 3d, the PRDII-NRE element is positioned 2 kb upstream. With each template, Dorsal, NF- κ B(p50/p65), and p65 homodimers (where tested) activated transcription above that elicited by endogenous activators bound to the TK promoter (lines 3, 5 and 7). Moreover, with the templates that bore a wild-type NRE (Figs 3a, b and d), Dorsal,

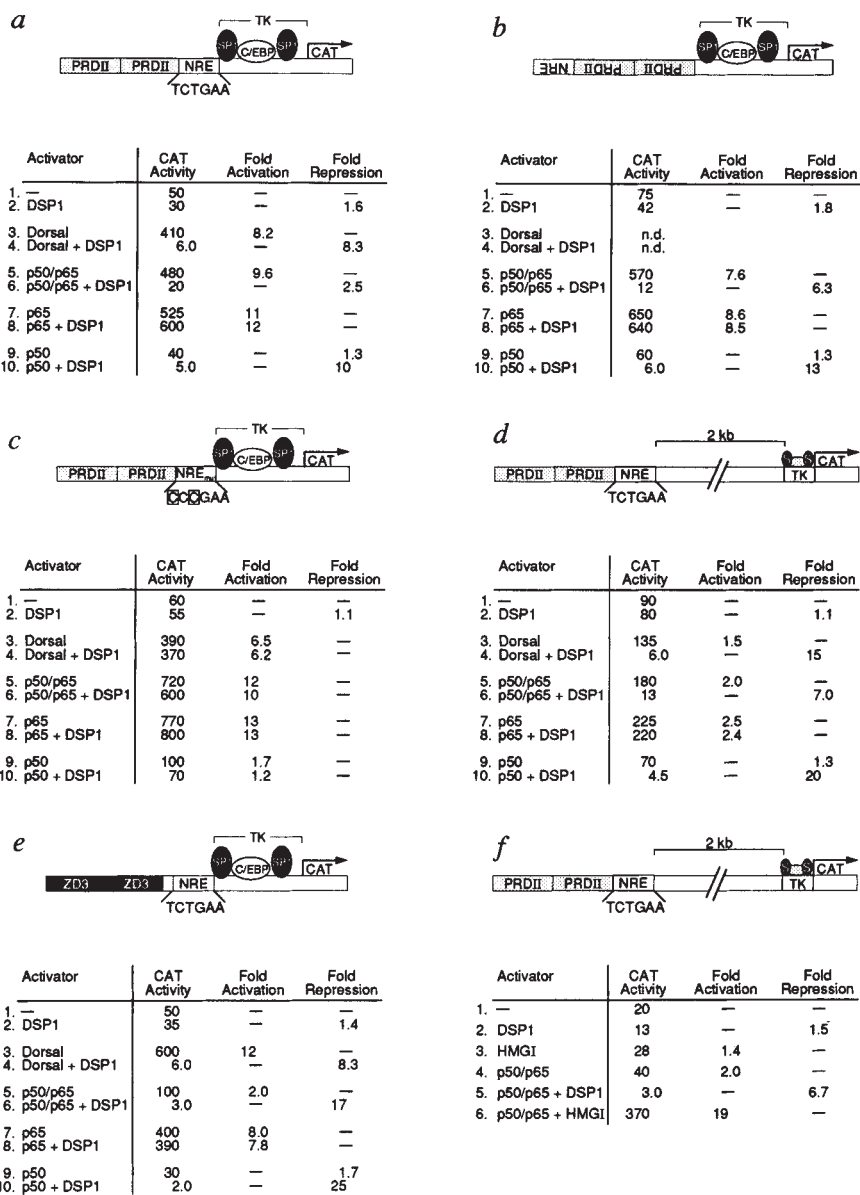
FIG. 3 DSP1 conversion of Dorsal and NF- κ B to repressors. The indicated reporters bear two PRDII sites and a wild-type or a mutant NRE or two ZD3 sites plus the adjacent NRE upstream of an activating element taken from the TK promoter. This element binds the endogenous transcriptional activators Sp1 and a C/EBP-like protein. These reporter plasmids were cotransfected into HeLa cells with mammalian expression vectors encoding the Rel family members, DSP1 and HMG I as indicated. *a*, The NRE is adjacent to the TK promoter; *b*, same as in *a* but the reporter plasmid bears the PRDII-NRE element in the opposite orientation; *c*, same as in *a* but the NRE contains the indicated base pair substitutions; *d*, same as in *a* but the PRDII-NRE element was cloned 2 kb upstream of the TK promoter; *e*, same as in *d* but the reporter was also cotransfected with a plasmid directing expression of HMG I; *f*, the reporter construct contains an oligonucleotide bearing two copies of the ZD3 element adjacent to an NRE found in the *zen* promoter and was cloned upstream of the TK promoter.

METHODS. The wild-type and the mutant oligonucleotides were cloned at the *Bam*HI site (*a-c*, *f*) of the pLCAT2 vector³⁰ or at the *Sma*I site (*d*, *e*). Transfections were carried out as described in Fig. 2. The expression vector for HMG I was constructed by inserting the open reading frame of HMG I at the *Bam*HI site of the mammalian expression vector pCDNA1 Amp (Invitrogen). The oligonucleotide GGGAAACAGTGGGAAAC-CAAGCTCTGAA containing two copies of the ZD3 site (underlined) and the adjacent NRE (italics) was cloned at the *Bam*HI site of the pLCAT2 vector.

p50 homodimers and NF- κ B, where tested, worked as repressors in the presence of DSP1. For example, Dorsal activated the reporter some eightfold above the level elicited by the endogenous activators bound to the TK promoter (Fig. 3*a*, line 3); the addition of DSP1 reduced transcription some 70-fold to a level eight-fold below that seen in the absence of Dorsal and DSP1 (Fig. 3*a*, line 4). When moved 2 kb upstream, Dorsal-DSP1 repressed the endogenously activated reporter to an even larger extent (15-fold, Fig. 3*d*, line 4). In contrast, activation by p65 homodimers was unaffected by the presence of DSP1 (lines 7 and 8). A mutant NRE, changed in two positions, failed to convey the repressive effect (Fig. 3*c*). Similar results were obtained with a reporter bearing two Dorsal binding sites⁵ (ZD3) and the NRE from the *zen* promoter (Fig. 3*e*), or with a reporter bearing the entire *zen* promoter VRE¹⁰ (data not shown). In contrast to its effect in mammalian cells, the Dorsal-DSP1 complex does not work as an upstream repressor in yeast. Thus in yeast, coexpression of Dorsal and DSP1 had no effect on transcription driven by the activators GCN4 or PPR1 bound to sites located between the VRE and the start site of transcription (data not shown).

Unlike DSP1, HMG I(Y), an HMG protein lacking HMG boxes^{9,18}, helped NF- κ B to activate transcription (Fig. 3*f*, compare lines 4 and 6), consistent with previous experiments⁹. The difference between CAT activity in the presence of the helper HMG I(Y) and the corepressor DSP1 was more than 120-fold (Fig. 3*f*). We imagine that a homologue (hDSP1) must fulfil DSP1's function in human cells. Neither of the widely expressed HMG box proteins hHMG1 or hHMG2 convert Rel activators to repressors, nor do they inhibit activation by Rel proteins (unpublished). hDSP1 must therefore be an unidentified HMG-like protein.

In vitro, recombinant DSP1 stimulated binding of recombinant NF- κ B (p65/p50) to an oligonucleotide bearing two



PRDII sites and an NRE (Fig. 4*a*). Moreover, DSP1 stimulated binding by p50 homodimers (Fig. 4*b*), but not by p65 homodimers (Fig. 4*c*). p50 homodimer binding was increased about ten-fold, and NF- κ B binding about three-fold (compare lanes 7–12 in Fig. 4*a*, *b*). These results parallel the effects of DSP1 on the activities of NF- κ B, p50 homodimers and p65 homodimers observed *in vivo*. It is therefore unlikely that the effect of DSP1 on Rel proteins is indirect.

Current views of repressor function suggest that repression can result from interactions of a 'repressing region' with an activator or with the transcriptional machinery^{1,23}. In Dorsal, the sequences required for DNA binding, transcriptional activation and DSP1-dependent repression are present on one polypeptide. In NF- κ B, however, activating sequences are found on p65, but not on p50, whereas the sequences required for DSP1-dependent repression are found on p50 and not on p65. We do not know whether the putative repressing region detected in our experiments is carried on the Rel component (p50 or Dorsal) or on DSP1.

We have not yet shown that DSP1 is the Dorsal corepressor in fly embryos, and other proteins in addition to DSP1 may ordinarily be involved in forming the repressing complex in the ventral region of the embryo. Indeed, mutations lying outside the Dorsal-NRE sites in the *zen* VRE can abolish repression^{24,25},

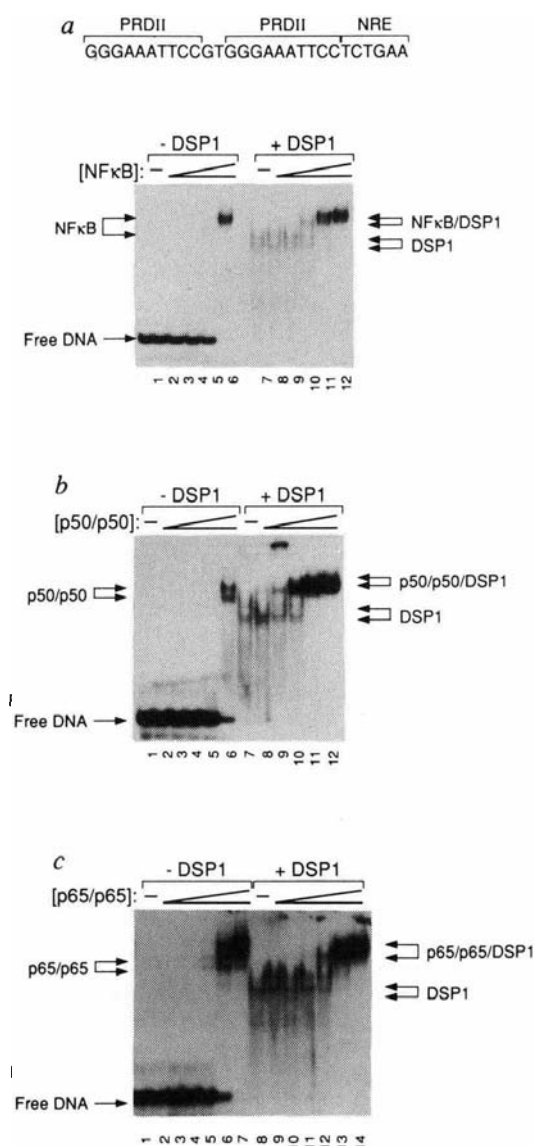


FIG. 4 Cooperative DNA binding by DSP1 and NF- κ B. **a**, Cooperative binding of DSP1 with NF- κ B (p50/p65 heterodimer) to an oligonucleotide containing two PRDII sites and the NRE. Lane 1 contains only the DNA probe. The concentration of NF- κ B in lane 6 is just enough to shift all the free probe. Lanes 2–6 contain increasing amounts of NF- κ B in threefold steps. Lane 7 contains 90 nM of DSP1, a concentration just enough to shift all the probe. Lanes 8–12 contain the same amounts of NF- κ B as lanes 2–6, but in the presence of a constant amount of DSP1 (90 nM). The concentration of NF- κ B required to produce the protein–DNA complex shown in lane 6 is threefold higher than the concentration required to produce a protein–DNA complex in the presence of DSP1 (lane 11). The locations of the free probe, the DSP1–DNA complex, the NF- κ B–DNA complex, and the slower migrating NF- κ B/DSP1–DNA complex are indicated. **b**, Cooperative binding of DSP1 with p50 homodimers (p50/p50). Electrophoretic mobility shift assays were performed as in **a**, with p50 homodimers in place of NF- κ B, except that the concentration of p50 homodimers required to produce the protein–DNA complex shown in lane 6 is ninefold higher than the concentration required to produce a protein–DNA complex in the presence of DSP1 (lane 10). The locations of the p50/p50–DNA complex and the slower migrating p50/p50–DSP1–DNA complex are indicated. **c**, Non-cooperative binding of DSP1 with p65 homodimers (p65/p65). Electrophoretic mobility shift assays were performed as in **a** with p65 homodimer in place of NF- κ B. Lane 1 contains only the DNA. The concentration of p65 homodimer in lane 7 is just enough to shift all the free probe. Lanes 2–7 contain increasing amounts of p65 homodimer in threefold steps. Lane 8 contains 90 nM of DSP1. Lanes 9–14 contain the same amounts of p65 homodimer as lanes 2–7, but in the presence of a constant amount of DSP1 (90 nM). The concentration of p65 homodimer required to produce the protein–DNA complex in lane 6 is the same as the concentration required to produce a protein–DNA complex in the presence of DSP1 (lane 13). The locations of the p65/p65–DNA complex and the more slowly migrating p65/p65/DSP1–DNA complex are indicated. **METHODS.** An NdeI site was introduced at the ATG of DSP1 by site-directed mutagenesis to make pSK–DSP1–Nde. DSP1 was subcloned into GEM11ZF (Promega) as a HindIII/NotI fragment and then cloned upstream of the phage T7 promoter by inserting DSP1 as an NdeI/XhoI fragment into pET16b (Novagen) which contains 10 histidines immediately downstream of the ATG. The resulting expression vector, pJBE18, was transformed into BL21 (DE3) pLysE, and DSP1 was induced at an optical density at 600 nm of 0.6 for 2 h with 1 mM isopropylthiogalactoside. DSP1 was purified by Ni²⁺ affinity chromatography as previously described²⁸. Partially purified DSP1 was then applied to a Fast S column (Pharmacia). Peak fractions were pooled and judged to be approximately 70% pure. The apparent relative molecular mass of DSP1 as calculated by SDS–PAGE was 49,000. NF- κ B was purified as previously described⁹. Electrophoretic mobility shift assays were performed on 20 μ l DNA-binding reactions in 10 mM Tris (pH 7.4), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.1% NP40, 8% glycerol, 1 mg ml⁻¹ BSA with approximately 10 fmol of labelled oligonucleotide. Reactions were incubated at 25 °C for 10 min and then samples were fractionated on a 4.5% polyacrylamide gel (containing 45 mM Tris base, 45 mM boric acid, 1 mM EDTA and 1% glycerol). Fractionation of DSP1 over a Superdex 200 (Pharmacia) gives $M_r \sim 100,000$ suggesting that DSP1 is a dimer in solution (not shown). The apparent dissociation constant of DSP1 for the depicted oligonucleotide was calculated by gel shift analysis as 10 nM (not shown). A similar value has been reported for hSR γ ³¹.

and the high concentrations *in vivo* of Rel protein and DSP1 obtained here may have overcome the need for such additional proteins.

The Rel-binding site NRE, a switch element, is conserved in flies and humans but has a very different role in the two cases described here. If DSP1 works in flies as it does in mammalian cell culture, a gradient of Dorsal formed against a constant background of DSP1 will lead to asymmetric expression of both activated and repressed targets. In humans, however, NRE-dependent repression may help to suppress basal transcription of the IFN- β gene, a desirable effect given the toxicity of interferon. □

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p16 and familial melanoma

It remains unclear just how big the latest player in the tumour-suppressor league will turn out to be. But p16 is not about to go away.

CHROMOSOME 9p21 is often lost or otherwise disrupted in a variety of human tumours¹. Typically, this sort of observation leads to the prediction of an incumbent tumour suppressor, and sure enough a candidate has been recently identified^{2,3}. *CDKN2* (or *MTS1* for multiple tumour-suppressor) maps to 9p21 and encodes a previously identified protein, p16, which is known to bind and inhibit the normal activity of the cyclin-dependent kinase (CDK) 4. Because the CDK family of proteins promote normal cell-cycle passage, and hence cell proliferation and growth, an inhibitor of this process, such as p16, is a plausible tumour-suppressor candidate. But it has emerged that whereas *MTS1* deletions are common in tumour cell lines they are much less common in primary tumours⁴, the implication being that many of the *in vitro* mutations might simply represent cell-culture artefacts.

The latest chapter in this story is to be found in three papers in the September issue of *Nature Genetics*⁵⁻⁷. In the first, Hussussian *et al.*⁵ report six disease-related *MTS1* germline mutations in nine of the 18 familial melanoma families they analysed. They also show that these mutations are in 33 of the 36 melanoma cases within these families, and are only found in families linked to the 9p21 locus and not in families linked to the other familial melanoma locus at 1p36. This is strong evidence that, despite some genetic heterogeneity, *MTS1* is a familial melanoma gene. And it might have been enough to silence the sceptics but for the second study by Kamb *et al.*⁶, which shows that, of eight American and five Dutch 9p-linked familial melanoma families, only two have what might be causative mutations of *MTS1*.

Nonetheless the two papers do permit some guarded conclusions to be drawn⁸. Both groups demonstrate that some 9p-associated families have mutations of *MTS1* although in others such mutations do not appear to be important. Of course mutations might have been missed (both

groups restricted their screen to coding or splice sequences and not all affected family members were analysed); or perhaps a second 9p21 gene might be involved. Still, for the time being we have an apparent contradiction. *MTS1* clearly has a role in familial melanoma, but is it playing the lead or merely a walk on part? The third paper⁷, a study of pancreatic adenocarcinomas, bears on this question.

Pancreatic adenocarcinomas are unusually aggressive tumours, which often show loss of the *p53* and *DCC* (deleted in colon cancer) genes⁹. Caldas *et al.*⁷, now present their results of screening a total of 27 such tumours. They examined ten cell lines, but also generated xenograft transplants in nude mice for a further 27 pancreatic adenocarcinomas. They report homozygous deletions of *MTS1* in 41%, sequence changes in 38% and loss of at least one allele in 22 of the 26 (85%) informative cases. Importantly, they were able to confirm many individual observations because often more than one xenograft was derived from the same primary tumour (either at several sites within one animal or in different animals), thus ruling out artefacts of the grafting procedure. Furthermore, comparison to normal tissue equivalents was particularly useful with regard to the sequence changes in that it confirmed that they were genuine somatic mutations.

These results clearly lend general support to the importance of p16 in cancer. Interestingly, the Dutch families studied by Kamb *et al.*⁶ also have a high incidence of pancreatic adenocarcinoma, a cancer for which Caldas *et al.* have demonstrated a very high frequency of *MTS1* mutations. Perhaps more attention should be turned to pancreatic adenocarcinoma pedigrees in an attempt to discover whether *MTS1* mutations are causative in pancreatic adenocarcinoma and to what extent this explains the *MTS1* mutations in the Dutch pedigrees. Who knows, this line of study might even bring down two birds with the same stone.

Also in this month's *Nature Genetics*, a team from the National Institutes of Health and Cornell Medical Center describes work with the first four patients to have received the cystic fibrosis (CF) gene into their lungs¹⁰. This paper provides a frank account of the likely safety of an adenoviral approach to CF gene therapy, and is all the more informative because one of the patients developed a local and systemic inflammatory

syndrome. This patient received the highest dose (2×10^9 plaque-forming units) of the CFTR (cystic fibrosis transmembrane regulator) recombinant adenovirus, administered, via a catheter, to the lower lobe bronchus of one lung and a second dose to the nasal epithelia. The patient suffered from headaches, fever, fatigue, tachycardia, some hypotension and breathing difficulties associated with significant levels of serum interleukin-6. These symptoms were treated and resolved by day 14, and there were no long-term side-effects during the one-year follow-up period.

It is encouraging that no complementation, recombination or shedding of the vector was seen; nor was there a rise in neutralizing antibodies (all patients had pre-existing anti-adenoviral antibodies), although an increase in serum IgE titre was noted in two patients. With regard to expression, CFTR messenger RNA was found in nasal epithelial samples of only one of the four patients, and no expression was seen in bronchial samples from the three patients tested. CFTR protein was found in nasal and bronchial epithelia in one patient.

This study was not intended to address issues of efficacy, and indeed few conclusions can be drawn on that score. Nevertheless, it is informative. It establishes an upper limit for the adenoviral human dose range, valuable information given that animal models may not help much in drawing up guidelines for human trials. Another lesson is evident from the fact that the patient who received the lowest viral dose (2×10^6 plaque-forming units) demonstrated CFTR expression in airway epithelia. This dose was 1,000 times less than that which caused the clinical syndrome in another patient. So although adenoviral-mediated CF gene therapy still has quite some way to go, its many critics have been given pause for thought.

Adrian J. Ivanson

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Also in this month's *Nature Genetics*: mutations in the fibroblast growth factor receptor-2 gene cause Crouzon syndrome; the length of uninterrupted CGG repeats determines *FMR1* instability; two new transcripts define a large imprinted domain in Prader-Willi syndrome; and 506 STS markers on a radiation hybrid map of chromosome 11.

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BIOTRONICS introduces a new, compact **instrument for PCR quantitation** (*Reader Service No. 100*). The instrument, the AG-9600 AmpliSensor Minitizer, is said to combine a 96-well format with the quantitative resolution of a kinetic PCR. The system



PCR quantitation device.

analyses the extent of PCR reaction by detecting the ongoing change in fluorescence signal. The need for post-amplification separation procedures such as electrophoresis, affinity capturing and hybridization have been eliminated with this system. The Minitizer is controlled by Windows-based software that also monitors the reaction and reports the results. An automated liquid dispensing facility is an optional extra.

The **primer and cover oil remover system** from Amresco provides a means of removing oligonucleotide primers and mineral oil from PCR reactions (*Reader Service No. 101*). PCR products (of greater than 250 base pairs) can be purified in about 15 min, without phenol, resins or glass beads, says Amresco. Stated primer removal is about 96 per cent. The purified DNA is then suitable for electrophoresis, cloning, restriction digestion and DNA sequencing. Amresco's system contains sufficient reagents for the purification of amplified DNA from 100 samples.

■ Isolation

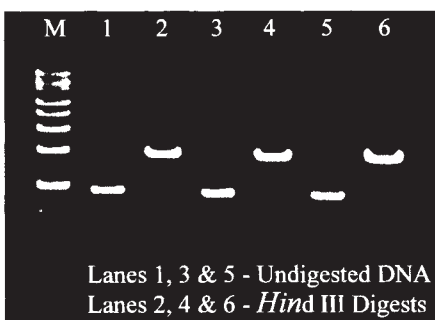
Pro-Cipitate LIS is a new **reagent for the isolation of DNA** that enables the pre-processing of DNA and RNA in standard enzyme modification and amplification protocols (*Reader Service No. 102*). Manufactured by Affinity Technology but available through Bionostics, this polymer (which is insoluble in water) has a configuration that enables it to dehydrate protein molecules causing precipitation.

NATURE • VOL 371 • 8 SEPTEMBER 1994

DNA and RNA remain unreacted, says the company, making it suitable for the preparation of plasmid DNA from bacterial lysates or for the isolation of genomic DNA and total RNA. Simple processing is required under nondenaturing conditions, with no requirement for evaporation or dialysis to remove residual reagents.

The Easy-DNA Kit is Invitrogen's new **kit for isolating genomic DNA** (*Reader Service No. 103*). It can be used with blood, cells or tissues, using small (1–100 µl of blood) or large (0.5–1 g of tissue) samples. The four-step procedure is said to take 90 min: just lyse, precipitate proteins, extract and precipitate the DNA to yield genomic DNA between 100–200 kilobases. This genomic DNA produces discrete PCR products and also DNA hybridization products in Southern blots. Invitrogen says that typical yields are 25 µg for 1 ml of blood; 150 µg for 50 mg of liver tissue.

Stratagene has a new method for isolating DNA that eliminates the need for phenol/chloroform extraction. The protocol in the ClearCut **miniprep kit** requires a microcentrifuge to process high-yield, small-scale preparations in 20 min (*Reader Service No. 104*). The kit can also be used to perform



Miniprep DNA isolated with Stratagene's ClearCut kit.

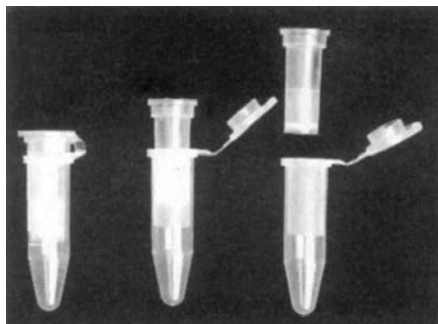
buffer exchanges and to remove primers and dNTPs from PCR products in 5 min, says Stratagene. It is used to bind the DNA, wash away all the reaction components and elute the DNA in water or tris-EDTA buffer. The extracted DNA can be used directly in restriction, ligation and sequencing reactions.

■ Extraction/filtration

A new kit from Oncor, which can be obtained through Alpha Laboratories, can be used to **extract DNA from paraffin blocks**

in 7 h (*Reader Service No. 105*). The protocol uses a non-organic extraction method, eliminating the need for xylene, phenol or chloroform disposal. It produces PCR products of 400 bases or more. Sufficient reagents are included to perform 20 DNA extractions containing $5 \times 5 \mu\text{m}$ -thick tissue sections per extraction.

Two new types of **spin filters** — one for microfiltration, the other for ultrafiltration — are now available from Integrated Sepa-



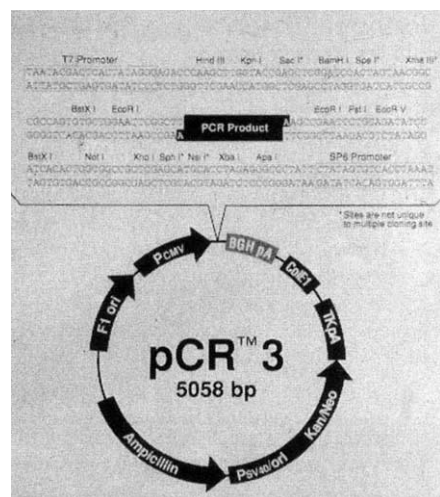
Spin filter selection.

ration Systems (*Reader Service No. 106*). Microfiltration spin filters are supplied in four membrane formats for DNA recovery from agarose, bead or resin-based protocols and deproteinizing DNA reactions. The ultrafiltration spin filters incorporate a low-binding, regenerated cellulose ultrafiltration membrane and are available with nominal molecular weight exclusions of 5,000, 10,000 and 30,000 daltons. Potential uses include cleanup of PCR and labelling reactions, protein or DNA concentration, and dialysis or desalting.

■ Kits

Using Amersham's new A-Tailing kit, it is possible to **insert a PCR-generated DNA fragment with any type of end base into T-vectors** (*Reader Service No. 107*). Previously, in order to use T-tailed vectors, PCR had to be performed using one of the thermostable polymerases (such as *Taq*), which preferentially adds a single adenosine base to the 3' end of the fragment. Now, Amersham says, any polymerase can be used for PCR product cloning. The A-Tailing kit includes all the reagents necessary to generate a 3' blunt end and then selectively to add the 3' adenosine residue, without the need for DNA precipitation. It is compatible with Amersham's pMOSBlue T-vector PCR cloning kit.

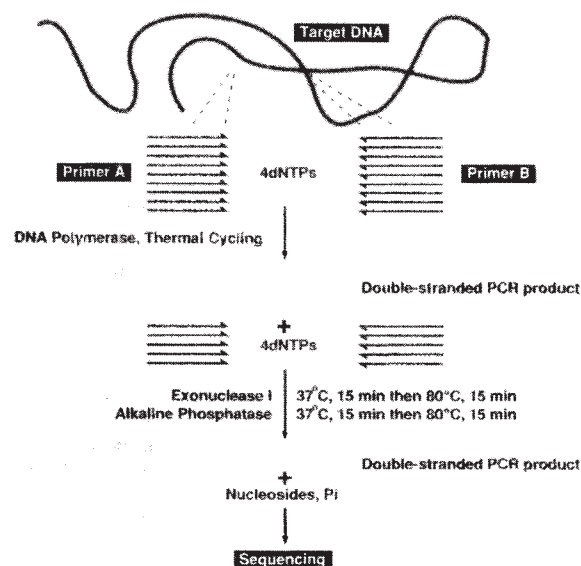
Invitrogen's Eukaryotic TA Cloning Kit has been developed for the **direct cloning of PCR products into a eukaryotic expression vector** (*Reader Service No. 108*). It is



Invitrogen's new TA cloning vector pCR3.

said to offer cloning efficiencies greater than 60 per cent and a one-step method that eliminates the need for complicated primer/linker strategies and inefficient blunt-end cloning techniques. The vector used in the kit, pCR3, contains the immediate-early cytomegalovirus promoter upstream of the PCR product insertion site. This, Invitrogen says, enables the user to proceed directly from PCR cloning to expression in mammalian cells without the need for tedious and time-consuming subcloning.

Sequence amplified gene fragments directly using the Sequenase **PCR product DNA sequencing kit**, which is available from Amersham (*Reader Service No. 109*). Following the amplification step, two enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, are added in series to remove



PCR product sequencing without purification.

single-stranded DNA/primers and nucleoside triphosphates, respectively, both of which can interfere with sequencing. After each step, the enzymes are inactivated by heat treatment before the Sequenase reaction. Amersham says that human genomic DNA and cloned DNA fragments can be sequenced after amplification using this kit; the process takes under 3 h, most of which is time taken up for thermal cycling. Many samples can be treated simultaneously.

SequiTherm is a cycle sequencing kit from Cambio for obtaining sequence data in ladders of up to 1,000 bases (*Reader Service No. 110*). The enzyme is also available separately. The protocol utilizes a thermostable DNA polymerase and multiple rounds of high-temperature DNA synthesis. Following heat denaturation of the template DNA, a large molar excess of sequencing primer is annealed to the template. The primer is then



See Cambio for cycle sequencing.

extended until incorporation of a dideoxynucleotide terminates the DNA synthesis. The denaturation, annealing and extension steps can be repeated 20–40 times to produce a stronger signal from the same amount of template. Apart from its ability to sequence very long strands, Cambio says that the protocol minimizes dropouts, requires less template and can use cosmids, PCR products and double-stranded plasmids.

The new GeneAmp EZ RNA PCR kit from Perkin-Elmer is specifically designed for rapid screening of samples using RNA PCR (*Reader Service No. 111*). The protocol utilizes both the reverse transcriptase and DNA polymerase activities of a single thermostable DNA polymerase, *rTth* DNA polymerase. The kit contains all the reagents necessary for reverse transcription of RNA to cDNA and subsequent amplification employing the GeneAmp PCR process using a single enzyme, *rTth* DNA polymerase, in a single reaction vessel, in a single buffer system. Use of a



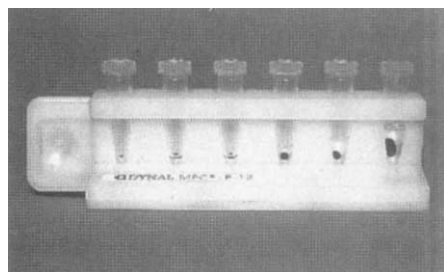
RNA PCR kit utilizing both the reverse transcriptase and DNA polymerase activities of a single thermostable DNA polymerase, *rTth* DNA polymerase. Targets present at 400 pg total cellular RNA except IL-1α(+) control (100 copies synthetic RNA), IL-1α(-) control (0 copies), HCV and HIV RNA (100 copies from plasma).

thermoactive and thermostable enzyme such as *rTth* DNA polymerase for both the reverse transcription and PCR steps is said to allow for increased specificity in primer binding, as well as alleviation of problematic secondary structures in the RNA template. A bicine, rather than the standard tris-HCl buffer, is used, enabling the buffering of both the metal and hydrogen ion concentration, which allows for efficient DNA polymerase activity at high temperature.

Magnetic means

NBL Gene Sciences has developed a new technology in applying activated magnetic particles to the purification of DNA. The Magpie **PCR purification kit** provides a rapid means of separating small-to-medium sized DNA fragments (300 base pairs to 6 kilobases) from reaction components such as dNTPs, NTPs and oligonucleotides (*Reader Service No. 112*). Double-stranded DNA is specifically bound to the ligand-coated beads, allowing contaminants to be removed during magnetic separation washes. The protocol is said to take about 40 min to complete.

Dynal has developed a new **magnet for use with PCR tubes** (*Reader Service No. 113*). The MPC-P-12 magnet holds 12 standard 0.5-ml PCR microtubes in two rows of six. It is fitted with a removable central magnet to facilitate the washing of Dynabeads, with high and low positions for optimum separations in large or small volumes, and allows the Dynabeads pellet to be con-



Dynal's magnet for PCR purposes.

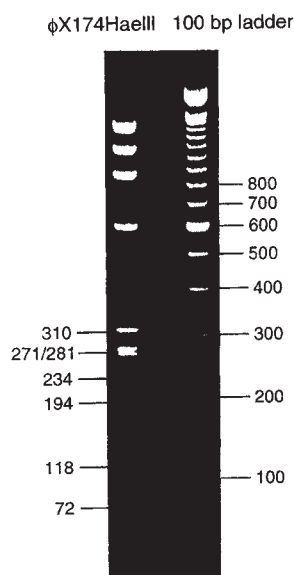
centrated down the tube wall. The magnet is intended for all PCR-based applications using Dynabeads — solid-phase DNA sequencing, RT-PCR and *in vitro* mutagenesis.

Gels

Agarose 3:1 high resolution blend is a blend of agarose for the resolution of small nucleic acid fragments and PCR products, obtainable through Amresco (*Reader Service No. 114*). The company says the agarose, which has been specifically designed for the resolution of DNA fragments of less than 1,000 base pairs, provides banding patterns without smearing or high background fluorescence. A gel strength of greater than 2,000 g cm⁻² makes it suitable for blotting applications. Each batch has been subjected to DNase/RNase testing.

Trevigen's TreviGel 500 is designed as an alternative to polyacrylamide for the separation of small DNA fragments obtained by PCR by horizontal gel electrophoresis (*Reader Service No. 115*).

The gel is intended for the resolution of fragments in the range of 50 to 1,300 bp. Trevigen is making the new separation matrix available in a powdered form or as 2 per cent precast gels ready for use in horizontal electrophoresis gel units. Precast gels measure 3 cm × 8 cm × 3 mm with five wells for loading the minimal DNA concentration of small PCR fragments needed for visualization.

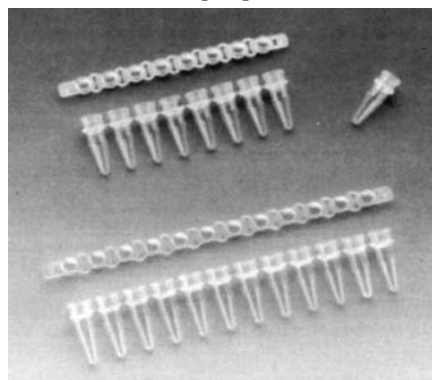


Typical electrophoretic results using 1.5% TreviGel 500 which is available from Trevigen.

The GeneAmp detection gel high resolution gel concentrate and loading buffer kit has been specifically developed to provide high resolution and fast run times for the separation of PCR products (*Reader Service No. 116*). Available from Perkin-Elmer and designed first for use with the company's AmpliFLP D1S80 amplification kit, the gel system is validated for forensic analysis using ethidium bromide or silver staining detection procedures. It is suitable for a range of research applications in addition to forensic testing. It contains a gel-forming solution composed of novel monomers and crosslinkers in a 5× concentrate, as well as loading buffer, and is said to resolve fragments in the range of 50 to 2,000 base pairs.

Tubes

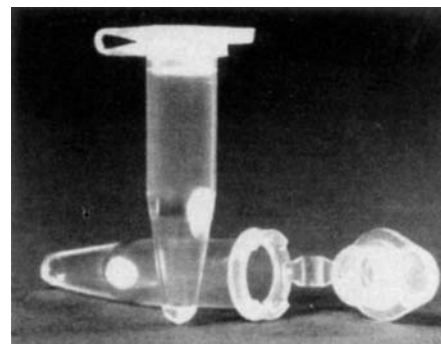
Robbins Scientific introduces Strip-Ease-12 strips of twelve thin-walled 0.2 ml PCR tubes and matching caps (*Reader Service*



Strips of PCR tubes.

No. 117). They complement the company's existing line of Strip-Ease-8 (eight-tube strips) and single tubes. All of these products are made from polypropylene, are available sterile or nonsterile in a choice of seven colours, and are certified DNase/RNase-free.

HotStart 100 storage and reaction tubes are 0.5-ml thin-walled PCR tubes, available from Molecular Bio-Products, which are supplied with a wax bead pre-adhered on the interior surface (*Reader Service No. 118*).



HotStart storage and reaction tubes.

Designed for use with PCR methods such as the 'hot start' technique, the wax is said to form a uniform convective vapour barrier to reduce significantly 'mispriming' and primer oligomerization. MBP says that the wax-mediated convective barrier allows for separation of the reaction components until the annealing temperature of the PCR reaction has been reached, thus increasing PCR specificity, sensitivity and accuracy. As a replacement for conventional mineral oil, HotStart reaction tubes are designed to cut down on evaporation, which can be beneficial when working with low-volume PCR reactions. The wax barrier seals the optimized PCR product or pre-aliquotted master mixes for safe and reliable long-term storage in a laboratory freezer (safe to a stated -20 °C) or even in a standard refrigerator. HotStart 50 and 100 tubes are now available for reactions of 20–50 µl and 50–100 µl, respectively. Tubes come presterilized and are certified to be DNase- and RNase-free.

Advanced Biotechnologies has launched a new line of 0.5-ml thin-walled reaction tubes with integral hinged caps (*Reader Service No. 119*). Compared to conventional tubes, the ultrathin walls are designed to allow maximum heat transfer with a reduc-

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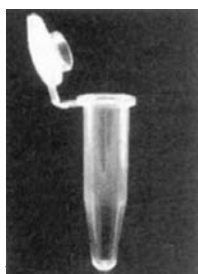
Primers and Probes
for DNA/RNA amplification
in Research and Diagnostics

Custom Oligonucleotide
Synthesis

Reader Service No.13

PRODUCT REVIEW

tion in lag time. The design makes the tubes particularly suitable for temperature-cycling procedures, improving uniformity and reducing cycle time. Made from high-grade polypropylene and manufactured under cleanroom conditions, Advanced Bio-technologies says that the tubes are guaranteed DNase- and RNase-free. Supplied in packs of 1,000 tubes.



Thermo-Tubes.

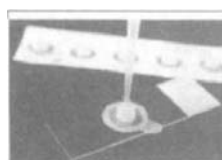
The **thin-walled tube**, multiply-Safecup, from Sarstedt is designed for thermal cycling procedures with sample volumes up to 100 μ l (*Reader Service No. 120*). Its design eliminates the need for mineral oil or wax overlays and, as such, sets out to reduce the number of steps involved. Aerosols are said to be reduced as the cap is screwed off rather than 'popped' off. The thin wall of the cup is also designed for optimal thermal exchange; its transparency aids pipetting. The Multiply-Safecup is intended for use with thermal cyclers without a heating lid.

■ *In situ* studies

A thermal cycler system that **standardizes and automates *in situ* procedures** is now available from Hybaid (*Reader Service No. 121*). Called OmniSlide, the device can handle up to 20 slides simultaneously. At the heart of the system is a high-capacity slide rack. Once loaded, samples remain in place through each stage of their *in situ* protocol. A special clip mechanism holds the slides in place and in thermal contact with the block. Integral to the design is a humidity chamber that maintains optimum conditions and reduces reagent loss from evaporation. For washing at uniform temperatures, the slide rack can be placed in a wash sleeve. When elevated temperatures of up to 70 °C are required, sleeves can be dropped into the wash module. This has two independently controlled chambers and is designed to utilize minimal wash volumes. If processing at room temperature is required, up to five sleeves or racks can be held upright in the ambient rack. Completing the system is a light-proof humidity chamber for light-sensitive incubation and signal detection.

Techné's **gene-well chambers** have been specifically designed for *in situ* applications (*Reader Service No. 122*). The chambers enclose 15-mm diameter specimens/tissue samples on a standard microscope slide to control loss of reagents due to evaporation during thermocycling procedures. Disposable and made of polypropylene, the chambers eliminate the need for nail polish, rubber cement, oil or wax, and replace coverslips. They have a self-adhesive rim that bonds to the glass microscope slide when heated. Techné says that the design of the

chamber ensures a quick thermal response, uses a small volume of reagent (50–75 μ l), eliminates air bubbles and are easy to seal. They can be used with the company's new *in situ* flat-plate Gene E thermal cycler, which has a capacity for up to four microscope slides, or any other make of slide thermal cycler/oven. Other features of the Gene E include a pause button for stopping a programme or for manually denaturing samples, an audible beeper for indicating the end of a programme and a parallel printer output.



Gene-Well chambers.

■ Thermal cyclers

Biometra offers a new **personal thermal cycler** that is based on Peltier technology (*Reader Service No. 123*). Design features



Biometra's personal thermal cycler.

of this new model include the ability to store up to 100 run routines, hot start, touch down and auto-restart functions, the ability to accommodate forty eight 0.2-ml or twenty 0.5-ml tubes, a bilingual LCD display, Centronics and RS232 interfaces. Optional extras include interchangeable heating blocks, a heated lid and in-tube control.

A wide range of **thermal cycler block sizes** for are offered with Barnstead/Thermolyne's new Amplitron I and II Thermal Cycler Dri Baths (*Reader Service No. 124*). Available in 36-, 48- or 96-well configurations, all offer compressor-based tech-



Amplitron thermal cyclers.

nology for the purposes of cooling. The environmentally-friendly refrigerant, HFC134A, is used. Up to 65 run programmes, containing 20 steps each, with 999 cycles, can be stored. Units have a heated lid to enable oil-free operation. This is an option on the 48-well, 0.5-ml unit; standard on the 96-well, 0.2-ml unit.

Idaho Technology's Air Thermo-Cycler is a **thermal cycler that uses high-velocity air as the heating/cooling medium** (*Reader Service No. 125*). This set-up means no oil overlays, no Peltier technology, water cooling or metal blocks. Specifications of this new thermal cycler, which can be obtained through Bio/Gene, include small sample volumes (5–50 μ l) in capillaries to reduce running costs, cycle times of 30–60 s, and a ramp rate of 10 °C s⁻¹ (peak rate). □

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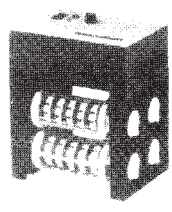
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